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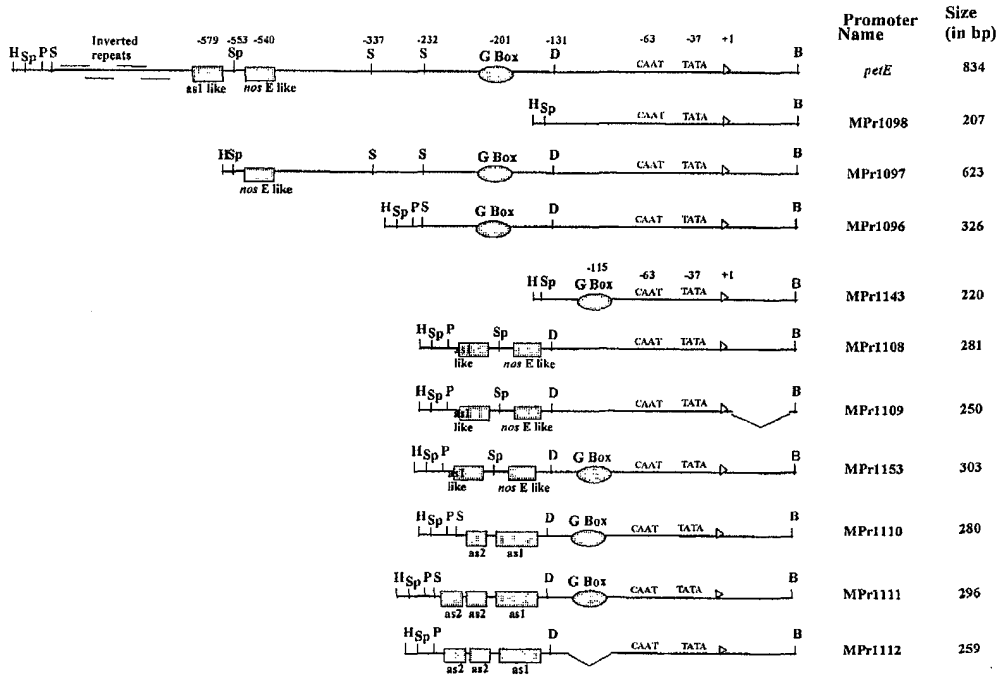
(71) Demandeur/Applicant:
MERISTEM THERAPEUTICS, FR

(72) Inventeurs/Inventors:
THEISEN, MANFRED, FR;
GRUBER, VERONIQUE, FR;
RANCE, IANN, FR

(74) Agent: GOUDREAU GAGE DUBUC

(54) Titre : PROMOTEURS CHIMERIQUES ASSOCIES AU PROMOTEUR PETE DE LA PLATOCYANINE PROVENANT DE PEA

(54) Title: CHIMERIC PROMOTERS BASED ON THE PLASTOCYANIN PETE PROMOTER FROM PEA

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(71) Applicant *(for all designated States except US)*: MERISTEM THERAPEUTICS [FR/FR]; Intellectual Property, 8 Rue des Frères Lumière, F–63100 Clermont Ferrand (FR).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: RANCE, Iann [FR/FR]; 54 Rue Proudhon, F–63000 Clermont Ferrand (FR). GRUBER, Veronique [FR/FR]; Residence Ste Madeleine, 44c Avenue Jean Jaurès, F–63400 Chamalieres (FR). THEISEN, Manfred [FR/FR]; 1 Rue Des Roseraies, F–63400 Chamalieres (FR).

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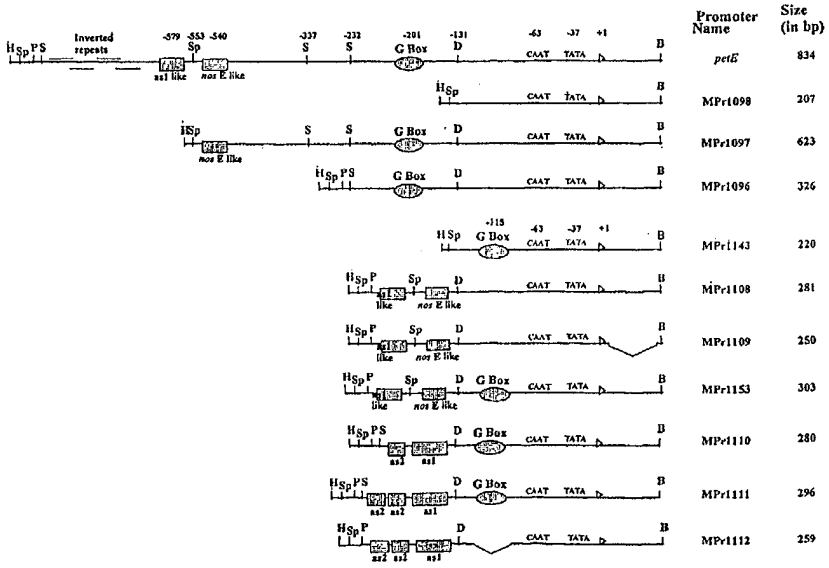


(57) Abstract

The present invention relates to chimeric promoters comprising at least one nucleic acid sequence derived from a promoter of the pea plastocyanin gene, the nucleic acid sequence preferably being derived from the petE promoter. The invention further relates to a method of production of such chimeric promoters, and expression cassettes, vectors, and transgenic plants containing them.

# CHIMERIC PROMOTERS BASED ON THE PLASTOCYANIN PETE PROMOTER FROM PEA

**[DESCRIPTION]**

The present invention relates to chimeric expression promoters, particularly suitable and adapted for use in the field of plant biotechnology.

In general, expression promoters are well known in the field of biotechnology and genetic manipulation. Insofar as plant biotechnology is more particularly concerned, the degree of expression of a gene coding for a polypeptide that it is desired to produce in a host cell is often dependent on the promoter used. The various promoters that are ubiquitously used are often limited to specific applications or particular cell tissues, simply because of their tissue specificity or expression strength. For example, one might cite the 35S promoter of the cauliflower mosaic virus as being a relatively strong promoter, compared to, say, the promoter originating from the *nos* gene, both of these promoters being more particularly used in the field of plant biotechnology. There thus exists at the present time a need for new and useful promoters that enable one to overcome the problems inherent in the promoters currently used up until now.

One attempt to solve this problem has been reported in the PCT application published under the number WO 97/20056, which describes increasing the degree of genetic expression through the use of "enhancers", (i.e. having a positive effect on the activity of the promoter), in known promoters. The "enhancing" nucleotide sequences are rich in A and T bases, the total amount of said bases coomprising more than 50% of the nucleotide sequence of the "enhancer". In particular, the applicants of this PCT application specify the use of an "enhancer" zone originating from the pea plastocyanin promoter.

The expressions used herein and in the claims are intended to have the following meanings :

- "nucleic acid" means DNA or RNA ;
- "nucleic acid sequence" means a single or double-stranded oligomer or polymer, of nucleotide bases read from the 5' extremity towards the 3' extremity, and includes self-replicating plasmids, genes, polymers of DNA or RNA, whether infectious or not, and also DNA or RNA whether functional or not. In the nucleotide notation used in the present application, and unless otherwise mentioned, the left extremity of a single stranded nucleotide sequence is the 5' extremity ;
- "derived nucleic acid sequence" means that the sequence derives directly or indirectly from the sequence to which reference is made, for example by substitution, deletion, addition, mutation, fragmentation, and/or synthesis of one or more nucleotides ;
- "promoter" or "promoting nucleic acid sequence" means a region of nucleic acid upstream of a start codon for translation and which is implicated in the recognition and binding of polymerase RNA and other proteins involved in transcription ;
- "plant promoter" means a promoter that is capable of initiating transcription plant cells ;
- "constitutive promoter" means a promoter capable of expressing nucleic acid sequences operably linked to said promoter, in all or nearly all the tissues of the host organism throughout the development of said organism ;
- "tissue specific promoter" means a promoter capable of epxressing, in a selective manner, nucleic acid sequences operably linked to said promoter, in certain specific tissues of the host organism ;
- "operably or functionally linked" means the linking of the promoter or the promoter nucleic acid sequence, to a nucleic acid sequence, or gene, to be expressed coding for a protein which it is desired to produce, in such a way that the promoter positively inflences or drives transcription of the linked nucleic acid sequence. It must be understood that that the promoter sequence may also include sequences which are

transcribed situated between the transcription start site and the translation start codon ;

- "nucleic acid sequence, or gene, to be expressed coding for a polypeptide that it is desired to produce" means a gene or nucleic acid sequence coding for a polypeptide, and preferably and exogenous or heterologous polypeptide, said exogenous polypeptide being more preferably a pharmaceutically, therapeutically or cosmetically active substance, such as a protein, enzyme, inhibitor, receptor, antibody, antigen, therapeutically active fragments thereof. Exemplary heterologous or exogenous therapeutically active substances that may be produced through expression of corresponding genes or nucleic acid sequences in a host cell are structural proteins, such as collagen, iron transfer proteins or transferrins, such as lactoferrin, blood derived proteins, such as hemoglobin, human serum albumin, erythropoietin, growth stimulating factors, proteolytic or anti-proteolytic enzymes, such as alpha-antitrypsine, hormones, secondary metoblites, digestive enzymes, such as gastric lipase, pancreatic lipase, trypsine, chymotrypsine, alcohol dehydrogenase, brain derived neurotrophic factors,cardiac stimulators or modifiers, and blood pressure control agents such as angiotensins;
- "expression cassette" means nucleotide sequences capable of driving expression of a nucleic acid sequence, or of a gene, coding for a polypeptide which it is desired to produce in a host organism which is compatible with such expression cassette sequences. Such expression cassettes generally comprise at least one promoter and a transcription termination signal, and optionally other factors necessary or useful for expression ;
- "vector" means expression systems, for example projectiles coated with DNA, nucleic acid based transport vehicles, nucleic acid molecules adapted for delivering nucleic acid sequences, and circular self-replicating autonomous DNA, for example plasmids, cosmids, phagemids, etc. If a recombinant micro-organism or recombinant cell culture is described as a

host for an "expression vector", this also includes circular extrachromosomal DNA (such as for example, mitochondrial or chloroplast DNA), DNA having been integrated into the host chromosome(s), the vector being either stably replicated by the cells during mitosis as an autonomous structure integrated into the host genome, or maintained in the nucleus or the cytoplasm of the host ;

- "plasmid" means a molecule of circular autonomous DNA capable of replication in a cell, and includes both "expression plasmids" and "non-expression plasmids". If a recombinant micro-organism or cell culture is described as a host for an "expression plasmid", this also includes molecules of circular extrachromosal DNA and DNA having been integrated into the host chromosome(s). If the plasmid is maintained by a host cell, the plamsid is either stably replicated by the cells during mitosis as an autonomous structure, or integrated into the host genome ;

- "heterologous sequence" or "heterologous nucleic acid sequence" means a sequence orginating from a source or species that is foreign to the environment, or if it originates from the same environment, has been modified with respect to its original form. The modification of the nucleic acid sequencecan occur for example through treatment of the nucleic acid with a restriction enzyme to generate a fragment of nucleic acid capable of beign operably linked to a promoter. The modificaiton can also occur through the use of techniques like site specific mutagenesis ;

- "box" means a nucleic acid sequence to which a regulatory function is attributed ;

- "like" means that the box and/or nucleic acid sequence with which this latter term is associated, comprises a certain sequence identity or homology with a known box and/or a known nucleic acid sequence indicated as a reference, and preferably a sequence identity of at least 50%, even more preferably a sequence identity of at least 75%, and most preferably a sequence identity of at least 90% with the reference sequence. The percentage of identity of the sequence is calculated on the basis of a comparison window of at least 6 contiguous nucleotide

bases. The determination of a comparison window can be carried out by using sequence alignment algorithms in order to determine an homology with the reference sequence, for example the local homology algorithm, the homology alignment algorithm, and the similarity search algorithm, these algorithms also existing as computer programs, and designated by names such as GAP, BESTFIT, FASTA and TFASTA. The percentage of sequence identity is obtained by comparing the reference sequence with the box and/or nucleic acid sequence using one or more of the abovementioned methods ;

- "situated" means the position on a nucleic acid sequence of an identified element, as for example a "box", a restriction site, or a codon having a particular function. The position that is given by a number refers to the position of the start of the element in the nucleic acid sequence, in the reading direction of the latter, i.e. in the direction 5'->3' with respect to the position of the transcription start site indicated as +1 ;
- "transgenic plant" means a plant having been obtained through the application of genetic manipulation techniques, and includes whole plants obtained by such techniques, their progeny, as well as plant organs, for example, but non limitatively, roots, stems, leaves and leaves, obtained by such techniques. The transgenic plants obtained according to the present invention can also have different degrees of diploidism, and can for example be polyploid, diploid, and haploid ;
- "propagule" means an accumulated mass or an association or an aggregation of structured or unstructured plant cells, capable of enabling the regeneration of a whole plant therefrom ; such associations can for example be in the form of explants, calli, stems, leaves, roots, cuttings, and even seeds.

The present applicant has taken a completely different approach to that taken by the applicant or the previously discussed PCT application. Serendipitously, the present applicant has managed to produce chimeric promoters which fill the need expressed previously, and in particular, enable an increase in the degree of expression of a gene, or a nucleic acid sequence operably

linked to said promoter, which codes for a polypeptide that it is desired to produce in a host cell, and preferably in a plant cell, with respect to the existing promoters currently in use. Furthermore, the present applicant has managed at the same time to produce a family of promoters in such a way as to enable the choice of a promoter most suited to a particular task or application or an environment in which it is intended to be used, and thus is able to control the degree of expression of a gene to be expressed, or a nucleic acid sequence operably linked to said promoter, that codes for a polypeptide which it is desired to produce in the host cell.

Consequently, one of the objects of the present invention is a chimeric expression promoter comprising at least one nucleic acid sequence derived from a promoter of the pea plastocyanin gene having the sequence identified under the number SEQ.ID01. Preferably, the chimeric promoter comprising a nucleic acid sequence derived from the promoter of the pea plastocyanin gene is selected from the group consisting of the sequences identified in the sequence listing under the numbers SEQ.ID02, SEQ.ID03, SEQ.ID04, SEQ.ID05, SEQ.ID06, SEQ.ID07, SEQ.ID08, SEQ.ID09, SEQ.ID10, and SEQ.ID11.

Furthermore, the present applicant has noticed that it is possible to build promoters according to the invention, and especially plant promoters, having an interesting promoter activity, whilst at the same having a minimum of regulatory boxes, and in particular at least one "G" box, a "CAAT" box and a "TATA" box, the only condition being that the "G" box be situated upstream of the other boxes, i.e. in the 5' region of the nucleic acid sequence, and that this "G" box be preferentially siutated at a certain distance, expressed in nucleotide bases, upstream from the other boxes. Thus, another object of the present invention is a chimeric expression promoter comprising a "G" box operably linked upstream of at least one "CAAT" box, one "TATA" box and a transcription initiation site (indicated by +1 in the figures). More preferably, the "G" box is situated between the positions - 225

and -65 with respect to the transcription initiation site (+1). Even more preferably, the "G" box is situated between the positions - 201 and - 115 with respect to the transcription initiation site (indicated by +1 in the figures). In the most preferred embodiments, the "G" box is situated either at position - 201, or at position - 115, with respect to the transcription initiation site (indicated as position +1 in the figures).

Advantageously, the "G" box is of plant origin. Preferably, the "G" box is obtained from a promoter of the pea plastocyanin gene. Even more preferably, the "G" box is obtained from the petE promoter of the pea plastocyanin gene.

According to a preferred embodiment of the promoters of the present invention, the promoters further comprise a "nos E like" box operably or functionally linked upstream of the "G" box.

According to another preferred embodiment of the invention, the promoters also comprise at least one "as1" or "as1 like" box operably or functionally linked to the "G" box, and preferably two or more such boxes, arranged either contiguously or separately, and most preferably four such boxes. These "as1" or "as1 like" boxes may be linked both upstream and downstream of said "G" box, and preferably are linked upstream thereof. In another preferred mode of the invention, one or more of the the "as1" or "as1 like" boxes can also be arranged in what is known as inverse order, that is, from the 3' to the 5' direction, and preferably in inverse repeat order.

According to one particularly preferred embodiment of the present invention, the promoters also comprise at least one "as2" box operably or functionally linked to the "G" box, and preferably two or more such boxes, arranged either contiguously or separately, and most preferably four such boxes. These boxes may also preferably be linked both upstream and downstream of said "G" box, and more preferably are linked upstream thereof. In another preferred mode of the invention, some or all of the boxes can also be arranged in what is known as inverse order, or as inverse repeats as described above.

Finally, and even more preferably, the chimeric promoters as described above comprise at least one nucleic acid sequence selected from the group consisting of the sequences identified in the sequence listing under the numbers SEQ.ID02, SEQ.ID03, SEQ.ID04, SEQ.ID05, SEQ.ID06, SEQ.ID07, SEQ.ID08, SEQ.ID09, SEQ.ID10, and SEQ.ID11.

Yet another object of the present invention is an expression cassette comprising at least one nucleic acid sequence derived from a promoter of the gene of the pea plastocyanin gene, operably or functionally linked to a gene or a nucleic acid sequence coding for a polypeptide that it is desired to express, said coding nucleic acid sequence being in turn operably or functionally linked to a transcription termination nucleic acid sequence, wherein the nucleic acid sequence derived from the promoter of the pea plastocyanin gene is selected from the sequences identified in the sequence listing under the numbers SEQ.ID02, SEQ.ID03, SEQ.ID04, SEQ.ID05, SEQ.ID06, SEQ.ID07, SEQ.ID08, SEQ.ID09, SEQ.ID10, and SEQ.ID11.

Another object of the present invention is an isolated promoter nucleic acid sequence, characterized in that the sequence is selected from the group consisting of the sequences identified in the sequence listing under the numbers SEQ.ID02, SEQ.ID03, SEQ.ID04, SEQ.ID05, SEQ.ID06, SEQ.ID07, SEQ.ID08, SEQ.ID09, SEQ.ID10, and SEQ.ID11. Such a sequence may be one obtained through deletion, substitution, addition of one or more nucleic acids to or from the sequence identified in the sequence listing under SEQ.ID01.

Yet still another object of the present invention relates to desoxynucleotide or desoxynucleoside blocks for the production of promoters of promoter nucleic acid sequences as identified previously. These blocks can be :

- building blocks or "directional" blocks, i.e. sequences that read in the same direction as the final promoter or promoter nucleic acid sequence sequence, which is from the 5′ end of the sequence towards the 3′ end of the sequence; and/or
- "guide" blocks, i.e. sequences whose ends include nucleotide

or nucleoside bases that complement with and overlap with the ends of the directional building blocks.

Thus, and most preferably, the directional building block corresponds to at least one sequence selected from the group consisting of the sequences identified in the sequence listing under the numbers SEQ.ID12, SEQ.ID13, SEQ.ID14, SEQ.ID15, SEQ.ID16, SEQ.ID17, and SEQ.ID18.

Furthermore, it is preferred to use the guide blocks corresponding to at least one sequence selected from the group consisting of the sequences identified in the sequence listing under the numbers SEQ.ID19, SEQ.ID20, SEQ.ID21, and SEQ.ID22.

Another object of the present invention is a vector comprising a promoter, or a promoter nucleic acid sequence, capable of initiating transcription of a gene or a nucleic acid sequence coding for a polypeptide that it is desired to produce, characterized in that the promoter or the promoter nucleic acid sequence corresponds to a chimeric expression promoter or to a promoter nucleic acid sequence as defined previously.

Preferably, the vector is selected from the group consisting in the binary vectors identified under the numbers pMRT1151, pMRT1149, pMRT1170.

Yet still another object of the present invention is a process for the production of a chimeric expression promoter or an isolated promoter nucleic acid sequence such as those defined previously, characterized in that it comprises the steps of :

- carrying out a ligation chain reaction, designated as LCR, to produce a continuous single stranded DNA molecule from at least one directional building block desoxynucleotide or desoxynucleoside sequence selected from the group consisting in the directional building blocks S1, S2, S3, S4, S5, S6, and S7 as identified in the sequence listing under the numbers SEQ.ID12, SEQ.ID13, SEQ.ID14, SEQ.ID15, SEQ.ID16, SEQ.ID17 and SEQ.ID18 respectively, and at least one desoxynucleotide or desoxynucleoside guide block for building said promoter nucleic acid sequence or promoter, the guide block being selected from the group consisting in the guide sequences G1, G2, G3, and G4

identified in the sequence listing under the numbers SEQ.ID19, SEQ.ID20, SEQ.ID21 and SEQ.ID22 respectively;
- carry out a PCR amplification on the single strand obtained from the preceding step, thereby enabling the production of a double stranded DNA corresponding to the chimeric expression promoter or tot the promoter nucleic acid sequence;
- optionally isolating the promoter or the promoter nucleic acid sequence.

Preferably, the desoxynucleotide or desoxynucleoside building blocks are phosphorylated before ligation. Even more preferably, the ligation is carried out in the presence of at least one DNA ligase in a thermocycle, under the following conditions :
- a first cycle of about one minute at about 94°C ;
- eight subsequent identical cycles, each one comprising the following steps :
- one minute at 65°C, one minute at 57°C, one minute at 52°C, one minute at 48°C, one minute at 43°C and ten minutes at 37°C.

Another object of the present invention is a transgenic plant having stably integrated into its genome at least one promoter or at least one promoter nucleic acid sequence as previously defined. Preferably, the transgenic plant is selected from dicotyledonous species, such as for example, and preferably, potato, tobacco, cotton, lettuce, tomato, melon, cucumber, peas, rapeseed, beetroot, and sunflower, and/or from monocotyledous species, such as for example, and preferably, wheat, barley, oats, rice and corn.

Another object of the present invention is a transgenic plant propagule, wherein propagule has the definition defined previously, and preferably the propagule is a seed.

Yet another object of the present invention is a cell containing a promoter or a promoter nucleic acid sequence as previously defined. Preferably, the cell is a procaryote or eucaryote cell, and even more preferably is a cell chosen from the group consisting of bacterial, insect, animal, human, fungal, algal, yeast and plant cells, and most preferably is a plant cell.

Among other preferred objects of the present invention, there is

also a process for the expression of a nucleic acid sequence, or gene, coding for a polypeptide that it is desired to produce, in a cell, characterized in that it comprises the following steps :
- transforming the cell with a vector comprising at least one promoter or at least one promoter nucleic acid sequence as defined previously, operably linked to a nucleic acid seqeunce or gene, coding for a polypeptide to be produced, itself operably linked to a transcription terminator signal ;
- culturing the cell in conditions enabling the expression of the nucleic acid sequence, or gene, coding for the polypeptide.

Preferably, the cell used in this method is a procaryote or eucaryote cell. Even more preferably, the celle is cell selected from the group consisting of bacterial cells, fungal cells, yeast cells, insect cells, animal cells, and plant cells, and most preferably is a plant cell.

Finally, another object of the present invention is a process for the production of a transgenic plant, or of a propagule, as defined previously, characterized in that the process includes the steps comprising :
- transforming a plant cell with a vector comprising at least one promoter or at least one promoter nucleic acid sequence as defined previously ;
- selecting the plant cell having integrated the promoter or promoter nucleic acid sequence into its genome ;
- propagating the transformed and selected plant cell, either through culture, or by regeneration of whole chimeric or transgenic plants.

**BRIEF DESCRIPTION OF THE FIGURES**

The present invention will be better understood through the following detailed description of several preferred embodiments given hereafter as best mode, but non-limiting examples, and by referring to the drawings in which :
- Figures I, II and III schematically represent the structures of comparative reference molecular constructs, enabling a comparison between the chimeric expression promoters of the present invention and said reference constructs. In Figure I,

the construction represented contains the reporter gene coding for ß-glucuronidase in the absence of any promoter sequence, and thereby serves as a negative control.

- Figure II schematically represents a construct containing the gene coding for ß-glucuronidase under the control of the CaMV double 35S promoter (d35S CaMV), also known as the 35S enhanced promoter (ep35S), which serves as a reference control for strong promoters ;

- Figure III represents a construct serving as an internal reference for transient expression experiments, and consists in the reporter gene coding for luciferase under control of the 35S CaMV promoter.

- Figure IV schematically represents the structure of several preferred embodiments of the chimeric promoters according to the present invention and derived from the whole promoter from the pea plastocyanin gene (petE prom), the latter also being represented. The promoters identified as MPr1098, MPr1097 and MPr1096 were obtained through enzymatic digestion of the whole promoter of the pea plastocyanin gene, whereas the promoters identified as MPr1108, MPr1109, MPr1110, MPr1111 and MPr1112 were obtained through ligand based PCR (lb-PCR). The promoters MPr1143 and MPr1153 were respectively obtained through deletion of the "as-2" and "as-1" boxes of promoter MPr1111, and through fusion of the "as-1 like" and "nos enhancer like" boxes of MPr1108 upstream of promoter MPr1143. All of the promoters produced were cloned into the vector pMRT1144 between the restriction sites PstI and BamHI in order to obtain transcriptional fusion with the reporter gene uidA which codes for ß-glucuronidase ;

- Figure V represents a graph comparing the relative promoter activities of the various constructs after transient expression in tobacco leaves. Three days after bombardment, the leaves were ground and the brute extract obtained clarified by centrifugation. The ß-glucuronidase and luciferase activities were measured by fluorimetry on an aliquot of brute extract, and the ratio GUS/LUC activities determined. The histograms

correspond to the average of the ratios for a given construct plus or minus Mean Standard Error (MSE) ;

- Figure VI represents schematic graphs of a comparison of chimeric promoter activity after stable transformation of tobacco plants. Samples were taken on all primary transformants at 2, 4, 6, 8 and 10 weeks after their transfer to greenhouse and β-glucuronidase activity measured from each sample, ponderated by the total protein content, producing a GUS activity in rlu/mg of protein. At each stage of developement, activities were sorted by decreasing order for each series of primary transformants and compared to each other.

In the various Figures, certain terms and expressions have the following meanings :

- uidA = a sequence coding for ß-glucuronidase ;
- IV2 = a patatin gene intron ;
- nos term = a terminator from the nopaline synthase gene ;
- 35S term = a terminator from the 35S RNA of CaMV ;
- B = the endonuclease restriction site BamHI ;
- E = the endonuclease restriction site EcoRI ;
- H = the endonuclease restriction site HindIII ;
- P = the endonuclease restriction site PstI ;
- Sp = the endonuclease restriction site SphI ;
- as-1 = the activating sequence 1 from the CaMV 35S promoter ;
- as-2 = the activating sequence 2 from the CaMV 35S promoter ;
- nos E = the activating box from the nopaline synthase promoter ;
- D = le site of restriction of l'endonucléase DraIII :
- H = the endonuclease restriction site HindIII ;
- P = the endonuclease restriction site PstI ;
- S = the endonuclease restriction site SpeI ;
- Sp = the endonuclease restriction site SphI ;
- CAAT = a "CAAT" box ;
- G = a "G" box ;
- TATA = a "TATA" box ;

- +1 = the transcription initiation site ;
- "like" means that the sequence is not 100% identical to the reference sequence from which the sequence obtains its name.

**DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

**Example 1**

**1. Comparative Constructs (controls)**

In order to enable a comparis of the chimeric expression promoters described in this application, the *uidA* gene coding for ß-glucuronidase (Jefferson and *al.*, 1986), and also containing the nucleic acid sequence of the IV2 intron of the gene *ST-LS1* from potato patatin (Vancanneyt and *al.*, 1990) (*uidA*-IV2) was placed under the control of one of the promoters and the terminator from the nopaline synthase gene (*nos* term). This construct was then transferred into *Agrobacterium tumefaciens* through cloning into the plasmid vector pGEM3Z sold by Promega Corp. (Madison, USA).

**1.1. Construction of a negative control identified as pMRT1144.**

In order to facilitate cloning, a plasmid vector derived from pGEM3Z, that only possessed the "*uidA*-IV2/*nos* term" sequences, in the absence of any promoter sequence, was made. This plasmid was called pMRT1144 and served as a negative control (Fig. I). The *uidA*/*nos* term sequences were introduced into the pGEM3Z plasmid, and the *uidA* sequence was placed under the control of the whole promoter of the pea plastocyanin gene and the nopaline synthase terminator, was isolated from 5 µg of pGA492-P*petE* plasmid. The latter plasmid was obtained by cloning, in the plamsid pGA492-P*em2*-*uidA*, the *petE* promoter from the pea plastocyanin gene obtained from the plasmid pKHn2 (Pwee and Gray, 1993) in place of the *em2* promoter (Gaubier and *al.*, 1993), originating from the bpI221-P*em2* plasmid. The bpI221-P*em2* plasmid was digested with 20 units each of the enzymes HindIII and EcoRI for an hour at 37°C. Then, the expression cassette "P*em2*/*uidA*/*nos* term" was separated by 0.8% agarose gel electrophoresis, electroeluted, precipitated in the presence of 1/10 volume of 3M sodium acetate at pH 4.8 and 2.5 volumes of

absolute ethanol at -80°C for 30 min, centrifuged at 12000 g for 30 min, washed in 70% ethanol, dried, resuspended in water and inserted in between the sites HindIII and EcoRI of the plasmid pGA492 (An, 1986). Ligation was carried out in the presence of 1.0 µl of T4 10X DNA ligase buffer (Amersham) and 2.5 units of T4 DNA ligase (Amersham) at 14°C for 16 hours. Viable and competent *Escherichia coli* DH5α bacteria were transformed (Hannahan, 1983). The plasmid DNA of the obtained clones, as selected on Luria-Bertani medium (LB, bactotryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l, Agar 15 g/l) supplemented with tetracycline (12 mg/l), was extracted according to the alkaline lysis method of Birnboim and Doly (Birnboim & Doly, 1983) and analyzed by enzymatic digestion.

Starting from the thus obtained pGA492-P*em2*-*uidA* plasmid, the promoter P*em2* was deleted via double digestion by HindIII and XbaI. The plasmid fragment of interest was separated by 0.8% agarose gel electrophoresis, electroeluted, precipitated in the the presence of 1/10 volume of 3M sodium acetate pH 4.8 and 2.5 volumes absolute ethanol at -80°C for 30 min, centrifuged at 12000 g for 30 min, washed in 70% ethanol, dried, and subjected to the action of a Klenow fragment of DNA polymerase I (New England Biolabs) for 30 min at 37°C according to the recommendation of the manufacturer. Then, it was deproteinized by extraction with a volume of phenol, then a volume of phenol : chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally one volume of chloroform : isoamyl alcohol (24:1 v/v), precipitated in the presence of 1/10 volume of 3M sodium acetate pH 4.8 and 2.5 volumes of absolute ethanol at -80°C for 30 min, then centrifuged at 12000 g for 30 min, washed in 70% ethanol, dried, and resuspended in water. Then it was dephosphorylized for 1h at 37°C with the help of 10 units of calf intestine alcaline phosphatase (Boehringer Mannheim) according to the recommendation of the manufacturer, deproteinized through extraction with one volume of phenol, then one volume of phenol : chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally one volume of chloroform : isoamyl alcohol (24:1 v/v), precipitated

in the presence of 1/10 volume 3M sodium acetate pH 4.8 and 2.5 volumes of absolute ethanol at -80°C for 30 min then centrifuged at 12000 g for 30 min, washed in 70% ethanol, dried then resuspended in water. The resulting plasmid was called pGA492ΔP*em2*.

In parallel, the *petE* promoter (818 base pairs), which corresponds to the promoter of the pea plastocyanine gene, was obtained from the plamsid pKHn2 by digestion with NcoI for 1 h at 37°C. The 828 bp promoter fragment was isolated on 0.8% gel agarose, electroeluted, precipitated in the presence of 1/10 volume of 3M sodium acetate pH 4.8 and 2.5 volumes of absolute ethanol at -80°C for 30 min, centrifuged at 12000 g for 30 min, washed in 70% ethanol, dried, resuspended in water, then subjected to the action of 5 units of Mung Bean nuclease (New England Biolabs) for 30 min at 30°C according to the recommendations of the manufacturer, deproteinized by extraction with one volume of phenol, then one volume fo phenol : chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally one volume of chloroform : isoamyl alcool (24:1 v/v), precipated in the presence 1/10 volume of 3M sodium acetate pH 4.8 and 2.5 volumes of absolute ethanol at -80°C for 30 min then centrifuged at 12000 g for 30 min, washed in 70% ethanol, dried, then resuspended in water. This promoter fragment was inserted into the plasmid pGA492ΔPem2, described previously, in the presence of 1.0 µl of T4 10X DNA ligase buffer (Amersham) and 2.5 units of T4 DNA ligase (Amersham) at 14°C for 16 h. Viable ad competent Escherichia coli DH5α bacteria, were transformed. The plasmid DNA of the obtained clones, selected on LB medium supplemented with tetracycline (12 mg/l), was extracted according to the alkaline lysis method and analyzed by enzymatic digestion. The resulting plamsid was designated pGA492-petE prom.

IN order to isolate the expression cassette "petE prom/uidA/nos term", 5 µg of the plasmid pGA492-petE prom were digested by PstI (at a 5' site on the plastocyanin gene promoter) and EcoRI (at a 3' site on the terminating sequence) for 1 h at 37°C,

subjected to 0.8% agarose gel electrophoresis and purified on a Qiaquick affinity column (Qiagen, Hilden, Allemagne) according to the manufacturer's recommendations. Meanwhile, 500 ng of pGEM3Z plasmid were simultaneously digested for 1 h at 37°C by EcoRI and PstI (restriction sites present in a multiple cloning site or polylinker), subjected to 0.8% gel electrophoresis, then purified on a Qiaquick affinity column.

Ligation was carried out with 50 ng of the vector pGEM3Z-PstI/EcoRI and 50 ng of the expression cassette "petE prom/uidA/nos term" for one night at 18°C in a reaction medium of 12 µl in the presence of 1.2 µl of T4 10X DNA ligase buffer (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs). Previously prepared viable and competent Escherichia coli DH5α, were transformed by mixing with the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB medium supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method and was analyzed by enzymatic digestion. The obtained plasmid was designated pGEM3Z-petE prom.

In order to insert the 192 bp IV2 intron originating from the potato patatin gene into the uidA coding sequence, an internal portion of this gene (a SnaBI / BstBI 710 bp fragment in pGEM3Z-petE prom) was excised then replaced by the equivalent sequence containing the IV2 intron (a SnaBI / BstBI 902 bp fragment). To accomplish this, the plasmid pGEM3Z-petE prom was digested for 1 h at 37°C by SnaBI (restriction site located at the +383 bp position upstream of the ATG initiator codon of the uidA gene) then for 1 h at 65°C by BstBI (restriction site located at the +1093 bp position). The plasmid comprising the 710 bp deletion was isolated by 0.8% agarose gel electrophoresis, then purified on a Qiaquick affinity column. The BstBI/SnaBI 902 bp fragment corresponding to the IV2 intron sequence followed by the uidA sequence stretching from position 383 to 1093 bp, was isolated and purified from the plasmid pSCV1.2-GI. This plamsid derives from plamsid pSCV1.2, which in turn derives from plamsid pSCV1 constructed by G.A. Edwards in

1990 according to the methods habitually used in cloning and well known to the skilled person. The binary plamsid pSCV1.2 was obtained by cloning the HindIII fragment bearing the expression cassette "35S prom / nptII / nos term" (Fromm and al., 1986) at the HindIII site in pSCV1. The expression cassette "35S prom / GUS-IV2 / 35S term" was obtained by digesting the plamsid p35S GUS INT with HindIII for 1 h at 37°C as described by Vancanneyt and al. (1990). The DNA fragment corresponding to the expression cassette was isolated on 0.8% agarose gel, electroeluted then precipitated in the presence of 1/10 volume of 3M sodium acetate at pH 4.8 and 2.5 volumes of absolute ethanol at -80°C for 30 min then, centrifuged at 12000 g for 30 min, washed in 70 % ethanol, dried and resuspended in water. The protruding 5' extremities of this fragment were blunted with the Klenow fragment of DNA polymerase I (New England Biolabs) for 30 min at 37°C according to the recommendations of the manufacturer, and the fragment was deproteinized by extraction with a volume of phenol, then a volume of phenol : chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally a volume of chloroform : isoamyl alcohol (24:1 v/v), precipitated in the presence of 1/10 volume of 3M sodium acetate at pH 4.8 and 2.5 volumes of absolute ethanol at -80°C for 30 min then, centrifuged at 12000 g for 30 min, washed in 70% ethanol, dried and finally ligated with 20 ng of the plasmid pSCV1.2 previously digested with SmaI for 1 h at 25°C, in the presence of 1.0 µl of T4 10X DNA ligase buffer (Amersham) and 2.5 units of T4 DNA ligase (Amersham) at 14°C for 16 h. Previously prepared viable and competent Escherichia coli DH5α bacteria were transformed. The plamsid DNA of the obtained clones, selected on LB medium supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method and analyzed by enzymatic digestion.

Five micrograms (5 µg) of pSCV1.2-GI plasmid were digested for 1 h at 37°C by SnaBI (restriction site located at the position +383 bp upstream of the ATG initiator codon of the uidA gene) then for 1 h at 65°C by BstBI (site located at the +1285 bp

position). The 902 bp fragment was isolated by 1.0% agarose gel electrophoresis, then purified on a Qiaquick affinity column. The ligation was carried out with 20 ng of vector pGEM3Z-petE prom BstBI/SnaBI and 80 ng of the 902 bp fragment BstBI/SnaBI, for 1 night at 18°C in a 10 µl reaction medium in the presence of 1.0 µl of T4 10X DNA ligase (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs). Viable and competent Escherichia coli DH5α bacteria, were transformed by half of the ligation reaction mixture. The plamsid DNA obtained from the clones, selected on LB medium supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method and was analyzed by enzymatic digestion. The obtained plasmid was designated pGEM3Z-petE prom/IV2.

In order to eliminate the promoter sequence corresponding to the 818 bp fragment (petE) of the plasmid pGEM3Z-petE prom/IV2, the latter was digested for 1 h at 37°C by BamHI then, for 1 h at 37°C by PstI, isolated by 0.8% agarose gel electrophoresis, then purified on a Quiaquick affinity column. The protruding 5' extremities of this plasmid were rendered blunt by using utilisant Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the recommendations of the supplier. The ligation was carried out with 10 ng of the thus modified plasmid for 1 night at 18°C in a reaction volume of 12 µl, in the presence of 1.2 µl of T4 10X DNA ligase (New England Biolabs) and 400 units of the enzyme T4 DNA ligase (New England Biolabs). Viable and competent Escherichia coli DH5α were transformed with half of the ligation reaction mixture. The plamsid DNA of the obtained clones, selected on LB medium supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method, analyzed by enzymatic digestion, and verified by sequencing according to the method of Sanger et al. (1977). The plasmid obtained was designated pMRT1144 (Fig. I).

**1.2. Construction of the positive control promoter MPr1092.**

In order to hava available a reference promoter sequence, the "double 35S" cauliflower mosaic virus promoter (CaMV D35S prom), was placed upstream of the uidA-IV2/nos term sequence. The

plasmid pMRT1092 (Fig. II) is a result of the following cloning steps :

To start with, the 192 bp IV2 intron of the potato patatin gene was inserted into the uidA coding sequence at the +383 bp position as described under section 1.1. A one microgram amount (1 µg) of bpI221 plasmid (Clontech, CA, USA) was digested for 1h30 at 37°C by SnaBI, then for 1h30 at 65°C by BstBI. The plasmid deleted of the 710 bp fragment was isolated by 0.8% agarose gel electrophoresis, then purified on a Quiaquick affinity column.

A twenty nanogram (20 ng) amount of bpI221 BstBI/SnaBI vector and 80 ng of the 902 bp BstBI/SnaBI fragment originating from pSCV1.2-GI as previously described, were ligated for 1 night at 18°C in a 10 µl reaction volume, in the presence of 1 µl of T4 10X DNA ligase (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs). Viable and competent Escherichia coli DH5α were transformed with hald of the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB medium supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method and analyzed by enzymatic digestion. The obtained plasmid was designated bpI221/uidA-IV2.

In a second step, the sequence of the CaMV35S promoter present in the bpI221/uidA-IV2 plasmid was replaced by the "CaMV D35S" sequence. This was achieved by digesting the bpI221/uidA-IV2 plamsid for 10h30 at 37°C with 10 units of HindIII, then the sticky ends were rendered blunt by the action of the Klenow fragment of DNA polymérase I (New England Biolabs) for 30 min at 37°C according to the recommendations of the manufacturer. After purification of the product of this reaction on a Qiaquick affinity column, the DNA was digesteed for une night at 37°C with 10 units of BamHI. The plasmid fragment, corresponding to the vector deleted of the 828 bp promoter CaMV 35S fragment, was isolated by 0.8% agarose gel electrophoresis, then purified on a Quiaquick affinity column.

CaMV D35S prom was obtained from the plasmid pJIT163D. This

derives from the plasmid pJIT163 which in turn derives from the plasmid pJIT160 (Guérineau and Mullineaux, 1993). The plasmid pJIT163 has an ATG codon between the HindIII and SalI sites of the polylinker. In order to delete this ATG and thereby obtain the plasmid pJIT163D, the plasmid DNA of pJIT163 was digested by HindIII and SalI, purified by 0.8% agarose gel electrophoresis, electroeluted, precipitated in the presence of 1/10 volume of 3M sodium acetate at pH 4.8 and 2.5 volumes of absolute ethanol at -80°C for 30 min, centrifuged at 12000 g for 30 min, washed in 70% ethanol, dried, subjected to the action of the Klenow fragment of DNA polymérase I (New England Biolabs) for 30 min at 37°C according to the recommendations of the manufacturer, deproteinized by extraction with a volume of phenol, then a volume of phenol : chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally a volume of chloroform : isoamyl alcohol (24:1 v/v), precipitated in the presence of 1/10 volume of 3M sodium acetate at pH 4.8 and 2.5 volumes of absolute ethanol at -80°C for 30 min, then centrifugé at 12000 g for 30 min, washed in 70 % ethanol, dried and finally ligated in the presence of 1.0 µl of T4 10X DNA ligase (Amersham) and 2.5 units of T4 DNA ligase (Amersham) at 14°C for 16 h. Viable and competent Escherichia coli DH5α bacteria were transformed. The plasmid DNA of the obtained clones, selected on LB mdeium supplemented with (50 mg/l), was extracted according to the alkaline lysis method and analyzed by enzymatic digestion.

Ten micrograms (10µg) of plasmid pJIT163D were digested for 10h30min at 37°C with 10 units of KpnI (site siutated in 5' of the promoter), then the sticky ends were rendered blunt by the action of 6 units of T4 DNA polymerase (New England Biolabs) for 30 min at 37°C according to the recommendations of the manufacturer. After purification of the product of this reaction on a Qiaquick affinity column, the DNA was digested for one night at 37°C with 10 units of BamHI. The resulting 761 bp DNA fragment, corresponding to the promoter CaMV D35S was isolated by 1.0 % gel agarose electropheresis, then purified on a Quiaquick affinity column.

The reaction mixture containing 10 ng of plasmid vector, 100 ng of the 761 bp fragment, 1.0 µl of T4 10X DNA ligase (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs) was subjected to ligation in 10 µl for one night at 18°C. Viable and competent Escherichia coli DH5α were trasnformed with half of the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method and analyzed by enzymatic digestion. The plasmid obtained was designated pMRT1092 (Fig. II).

**1.3. Description of the reference plasmid pCaMV35S1uc.**

The plasmid serving as an internal reference for transient expression is pCaMV35S1uc (Torrent et al., 1997) which contains the expression cassette of the luciferase reporter gene (luc) under the control of the RNA 35S Cauliflower Mosaic Virus promoter and terminator.

**Example 2.**

**Construction of plasmids containing deleted promoter sequences from the pea plastocyanine gene.**

The whole promoter of the pea plastocyanin gene (Last and Gray, 1989) corresponds to a sequence of 834 bp (SEQ. ID01) starting from position -771 bp to position + 63 bp, and in which several potential regulatory sequences have been identified (numbers given with respect to the 5' end towards the 3' end of the sequence, and with respect to the transcription initiation site +1, Fig. IV) :

- a series of inverted repeats, stretching from positions -734 to -607 bp,
- a 20 bp box (as-1 like) having a certain similarity to the activating sequence 1 (as-1) present in the CaMV 35S promoter, and stretching from position -579 to position -559 bp,
- a 21 bp box (nos enhancer like) having a certain homology to an activating sequence present in the promoter of the nopaline synthase gene of *Agrobacterium tumefaciens*, and stretching from position -540 to -519 bp,
- an 8 bp "G" box, stretching from position -201 to position

-193 bp,
- a 14 bp box, stretching from position -83 to position -69 bp, and having a certain similarity to type III boxes found in the promoters of higher plants,
- a "CAAT" box, at position -63
- a "TATA" box, at position \37
- the transcription initiation site +1 (position 1)
- a 5' untranslated region stretching from the +1 position to the 63 bp position.

The plasmid pGEM3Z-petE prom was obtained as described above in section 1.1 of example 1. It corresponds to the plasmid pGEM3Z containing the reporter gene uidA-IV2 under the control of the whole of the pea plastocyanin gene (petE prom, Fig IV, SEQ.ID01), and serves as a reference promoter for all of the constructions based on the plastocyanin promoter.
In order to study the effects of the different elements, deletions in the 5' region of petE prom were carried out by enzymatic digestion.

**2.1. Construction of the promoter MPr1097.**

The promoter MPr1097 derives from the petE promoter by a 5' deletion of the inverted sequence repeats, as well as the as-1 like box borne on the 212 bp Sph1 fragment (Fig. IV). In order to do this, five micrograms (5 µg) of the plasmid pGEM3Z-petE/IV2 were digested for 2 h at 37°C with 20 units of SphI enzyme, isolated by 0.8% agarose gel electrophoresis, then purified on a Qiaquick affinity column.
The ligation was carried out with 25 ng of the thus modified plasmid for one night at 18°C in a 10 µl reaction mixture, in the presence of 1 µl of T4 10X DNA ligase (New England Biolabs) and of 400 units of T4 DNA ligase (New England Biolabs). Viable and competent Escherichia coli DH5α cells were transformed with half the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method, and analyzed by enzymatic digestion. The plasmid obtained was designated pMRT1097 and the promoter sequence

designated MPr1097 (SEQ.ID02) was verified by sequencing.

**2.2. Construction of the promoter MPr1096.**

The promoter MPr1096 derives from the petE promoter by a 5' deletion of the inverted repeat sequences, the "as-1 like" elements and the "nos enhancer like" element borne by two SpeI fragments of 403 bp and 105 bp respectively (Fig. IV). In order to do this, 5 micrograms (µg) of plasmid pGEM3Z-petE /IV2 were digested for 2 h at 37°C with 20 units of SpeI enzyme, isolated by 0.8% agarose gel electrophoresis, and then purified on a Qiaquick affinity column.

The ligation was carried out with 25 ng of the thus modified plasmid for 1 night at 18°C in a 10 µl reaction mixture, in the presence of 1 µl of T4 10X DNA ligase (New England Biolabs) and of 400 units T4 DNA ligase (New England Biolabs). Viable and competent Escherichia coli DH5α cells were transformed with half of the ligation reaction mixture. The plamsid DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method, and analyzed by enzymatic digestion. The plasmid obtained was designated pMRT1096 and the promoter sequence MPr1096 (SEQ.ID03) was verified by sequencing.

**2.3. Construction of the promoter MPr1098.**

In order to obtain a reference minimal promoter sequence based on the petE promoter, the 207 bp promoter MPr1098 was constructed to contain only the "TATA" and "CAAT" boxes (Fig. IV).

This was accomplished by digesting five micrograms (5 µg) of the plasmid pGEM3Z-petE /IV2 for 2 h at 37°C with 1.5 units of DraIII enzyme (1 site present at position -128) and 15 units of PstI enzyme (1 site present in the 5' region of the promoter at position -759 bp). The plasmid thus deleted of the 631 bp fragment PstI/DraIII situated in the 5' region of the petE promoter was isolated by 0.8% agarose gel electrophoresis, then purified on a Quiaquick affinity column. The sticky ends of this fragment were rendered blunt by the action of 6 units of T4 DNA polymérase (New England Biolabs) for 30 min at 37°C according to

the recommendations of the manufacturer. After purification of the product of this reaction on a Qiaquick affinity column, ligation was carried out with 25 ng of the thus modified plasmid for 1 night at 18°C in a 10 µl reaction mixture, in the presence of 1 µl of T4 10X DNA ligase (New England Biolabs) and of 400 units of T4 DNA ligase (New England Biolabs). Viable and competent Escherichia coli DH5α cells were transformed with half the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method and analyzed by enzymatic digestion. The plasmid obtained was dsignated pMRT1098 and the promoter sequence MPr1098 was verified by sequencing (SEQ.ID04). This sequence corresponds to the minimal pea plastocyanin promoter (Fig. IV).

**Example 3.**

**Construction of plasmids containing chimeric promoter sequences.**

In addition to the promoters obtained by deletion of certain 5' regions, a series of promoters was synthesized that started out from the basic minimal promoter Mpr1098 using the lb-PCR technique which combines a ligation chain reaction, designated LCR (Barany, 1991) and producing a single stranded continuous DNA with the help of "directional" oligodesoxynucleotides, with a PCR reaction leading to a double stranded DNA product.

**3.1. Construction of the promoter MPr1108.**

The promoter MPr1108 (Fig. IV) was created by fusing the 72 bp sequence stretching from position -641 bp to position -569 bp of the petE promoter (SEQ. ID01), and bearing the "as-1 like" and "nos enhancer like" boxes, to the 187 bp minimal promoter sequence of MPr1098 (position -128 to +59 bp, SEQ.ID04) using the lb-PCR technique.

Continuous single stranded DNA was formed with the help of the following directional oligodesoxynucleotides :

- S1 =

5' TTCCCTTCAAACACATACAAATTCAGTAGAGAAGAAACTCATTACTCTTGAGAAACCTAG AGGATCCCCG 3' (SEQ.ID12)

- S2 =

5' CACAAAAACCCAATCCACATCTTTATCATCCATTCTATAAAAAATCACCTTCTGTGTGTC TCTCTTTCGA 3' (SEQ.ID13)

- S3 =

5' CATAATTTGAACACTCTGTGGCACATCTACATTATCTAAATCACATATTCTTCCACACAT CTTAGCCA 3' (SEQ.ID14)

- S4 =

5' GGAATCTGCAGTTGAACACGTACAAACTTACGTCATTTGTGCATGCAGAAGCATAGAGCT GAGCACACAATT 3' (SEQ.ID15)

One hundred picomole (100 pmol) of the S1, S2 and S3 oligodesoxynucleotides were 5' phosphorylated by the action of 15 units of kinase (Amersham) in the presence of 5 µl of 10X kinase (Amersham) and 500 pmol of ATP (Sigma), for 30 minutes at 37°C. The phosphorylated oligodesoxynucleotides were purified by extraction with a volume of phenol, then a volume of phenol : chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally a volume of chloroform : isoamyl alcohol (24:1 v/v), before being precipitated with 1/10 volume of 3M sodium acetate at pH 4.8 and 2.5 volumes of absolute ethanol at -80°C for 20 min then centrifuged at 16060 g for 30 min. The precipitated oligodesoxynucleotides were washed with 70% ethanol, dried, then resuspended in water at a concentration of 10 pmol/µl.

In order to link up the "directional" oligodesoxynucleotides, the following "guide" oligodesoxynucleotides were used :

- G1= 5' TGTGTTTGAAGGGAATCGAAAGAGAGACACA 3' (SEQ.ID18)
- G2= 5' GATTGGGTTTTTGTGTGGCTAAGATGTGTG 3' (SEQ.ID19)
- G3= 5' CAGAGTGTTCAAATTATGAATTGTGTGCTCAGC 3' (SEQ.ID20)

In order to carry out the LCR reaction, 10 pmol of the phosphorylated S1, S2, S3 and S4 "directional" oligodesoxynucleotides were ligated in the presence of 10 pmol of the G1, G2 and G3 "guide" oligodesoxynucleotides, 5 µl of Taq 10X DNA ligase (New England Biolabs) and 40 units of Taq DNA Ligase (New England Biolabs). The ligation reaction was carried out in a GeneAmp PCR System 9700 thermocycle (Perkin Elmer, Norwalk, USA). The reaction involved the steps consisting of one cycle for one minute at 94°C, and of 8 identical subsequent

cycles, each consisting in the following : one minute at 65°C, one minute at 57°C, one minute at 52°C, one minute at 48°C, one minute at 43°C, and finally ten minutes at 37°C. Next, the ligation reaction mixture was purified on a Qiaquick affinity column according to the recommendations of the supplier. Finally, PCR amplification of the obtained single stranded DNA was carried out in a GeneAmp PCR System 9700 thermocycle in the presence 100 pmol of each of the oligodesoxynucleotide probes 5' GGAATCTGCAGTTGAACACGT 3' and 5 CGGGGATCCTCTAGGTTTCT 3', 50 nmol of each of the dNTP, 10 µl Vent 10X DNA polymerase buffer (New England Biolabs), and 2 units of Vent DNA polymerase (New England Biolabs). The DNA was denatured for 5 min at 94°C, subjected to 25 cycles each consisting of a 30 second denaturizing step at 95°C, of a 30 second hybridizing step at 56°C, and of one minute of elongation at 72°C, then elongation at 72°C was continued for 5 min.

The DNA fragments of the reaction mixture were digested with 20 units of BamHI for 45 min at 37°C, then by 20 units of PstI for 1 h at 37°C, and finally purified on a Qiaquick affinity column. They were inserted into the plasmid pGEM3Z-petE /IV2 that had been digested with BamHI enzyme for 1 h at 37°C, then with PstI enzyme for 1 h at 37°C, and subjected to 0.8% agarose gel electrophoresis, purified on a Quiaquick affinity column, dephosphorylated for 1 h at 37°C in the presence of 12 µl of "buffer 3" 10X (New England Biolabs) and of 5000 units of caclf gut alkaline phosphatase (CIP, New England Biolabs), and finally purified on a Quiaquick affinity column. In order to carry out ligation, 25 ng of of the plasmid as treated above were contacted with 100 ng of the DNA fragments obtained by PCR, in the presence of 1.2 µl of T4 10X DNA ligase buffer (New England Biolabs) and 400 units T4 DNA ligase (New England Biolabs) for 1 night at 18°C. Viable and competent Escherichia coli DH5α cells were transformed with half of the ligation reaction mixture. The DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method, and analyzed by enzymatic digestion. Two

plasmids pMRT1108 and pMRT1109 resulting therefrom were sequence. The plasmid pMRT1108 contains the expected promoter (SEQ.ID05), whereas the plasmid pMRT1109 bears the promoter sequence MPr1109 (SEQ.ID06) which differs from MPr1108 by a deletion of 33 bp in the 5' untranslated region, 11 bp upstream of position +1 (Fig. IV).

**3.2. Construction of the promoter MPr1110.**

The promoter MPr1110 was created by inserting, at position -99 bp of the MPr1098 promoter (SEQ.ID04), a block of 18 bp containing a "G" box (stretching from positions -204 bp to -186 bp of the petE promoter, SEQ.ID01) and by fusing to this modified minimal promoter minimal a sequence of 44 bp from the RNA 35S cauliflower mosaic virus promoter (CaMV) containing the as-2 and as-1 elements (Lam, 1989 ; Lam and al., 1989) (Fig. IV). MPr1110 was synthesized by the lb-PCR technique.

The continuous single stranded DNA was formed with the help of the following "directional" oligodesoxynucleotides :

- S1 =

5' TTCCCTTCAAACACATACAAATTCAGTAGAGAAGAAACTCATTACTCTTGAGAAACCTAG AGGATCCCCG 3' (SEQ.ID12)

- S2 =

5' CACAAAAACCCAATCCACATCTTTATCATCCATTCTATAAAAAATCACCTTCTGTGTGTC TCTCTTTCGA 3' (SEQ.ID13)

- S5 =

5' CTGTGGCACATCTACATTATCTAAATCTAAGCCACGTCGGAGGATAACATATTCTTCCAC ACATCTTAGCCA 3' (SEQ.ID16)

- S6 =

5' CATGCTGCAGACTAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCATGCC ACT 3' (SEQ.ID17)

One hundred picomole (100 pmol) of the S1, S2 and S5 oligodesoxynucleotides were 5' phosphorylated with the help of 15 units of kinase (Amersham) in the presence of 5 µl of 10X kinase buffer (Amersham) and 500 pmol of ATP (Sigma), for 30 min at 37°C. The phosphorylated oligodesoxynucleotides were purified by extraction with a volume of phenol, then a volume of phenol :

chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally a volume of chloroform : isoamyl alcohol (24:1 v/v), before being precipitated with 1/10 volume of 3M sodium acetate at pH 4.8 and 2.5 volumes absolute ethanol at -80°C for 20 min then centrifuged at 16060 g for 30 min. Les precipitated oligodesoxynucleotides were washed in 70% ethanol, dried, then resuspended in water at une concentration of 10 pmol/µl.

In order to link up the "directional" oligodesoxynucleotides, the following "guide" oligodesoxynucleotides were used :

- G1= 5' TGTGTTTGAAGGGAATCGAAAGAGAGACACA 3' (SEQ.ID19)
- G2= 5' GATTGGGTTTTTGTGTGGCTAAGATGTGTG 3' (SEQ.ID20)
- G4= 5' TGTAGATGTGCCACAGAGTGGCATGCGT 3' (SEQ.ID22)

In order to carry out the LCR reaction, 10 pmol of the phosphorylated "directional" oligodesoxynucleotides S1, S2, S5 and S6 were ligated in the presence of 10 pmol of the "guide" oligodesoxynucleotides G1, G2 and G4, 5 µl of the Taq 10X DNA ligase buffer and 40 units of Taq DNA Ligase (New England Biolabs). The ligation reaction was carried out in a GeneAmp PCR System 9700 thermocycle (Perkin Elmer, Norwalk, USA). It was composed of a one minute cycle at 94°C, and of 8 identical cycles each comprising the following steps : 1 min at 65°C, 1 min at 57°C, 1 min at 52°C, 1 min at 48°C, 1 min at 43°C, and finally 10 min at 37°C. then, the ligation reaction mixture was purified on a Qiaquick column according to the supplier's recommendations.

Finally, PCR ammplification of the single stranded DNA obtained previously was carried out dans in a GeneAmp PCR System 9700 thermocycle in the presence of 100 pmol of each of the following oligodesoxynucléotide probes 5' CATGCTGCAGACTAGTGGATT 3', and 5' CGGGGATCCTCTAGGTTTCT 3', of 50 nmol of each of the dNTP, of 10 µl of Vent 10X DNA polymerase buffer(New England Biolabs), and of 2 units Vent DNA polymerase (New England Biolabs). The DNA was denatured for 5 min at 94°C, subjected to 25 cycles each composed of 30 sec of a denaturing step at 95°C, of 30 sec of a hybridization step at 56°C, and of 1 min of elongation at 72°C, then elongation at 72°C was continued for 5 min.

The DNA fragments of the reaction mixture were digested with 20 units of BamHI enzyme for 45 min at 37°C then with 20 units of PstI enzyme for 1 h at 37°C, and finally purified on a Qiaquick column. They were inserted into the plasmid pGem3Z-petE /IV2 which had been previously digested by BamHI enzyme for 1 h at 37°C and then by PstI enzyme for 1 h at 37°C, subjected to 0.8% agarose gel electrophoresis, purified on a Quiaquick affinity column, dephosphorylated for 1 h at 37°C in the presence of 12 µl of "Buffer 3" 10X (New England Biolabs) and of 5000 units of calf intestine alkaline phosphatase (CIP, New England Biolabs), and finally purified on a Quiaquick affinity column. In order to carry out the ligation, 25 ng of the plasmid treated as described above were brought into contact with 100 ng of the DNA fragments obtained by PCR, in the presence of 1.2 µl of T4 10X DNA ligase buffer (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs) for 1 night at 18°C. Previously prepared viable and competent Escherichia coli DH5α cells were transformed with half of the ligation reaction mixture. The DNA from the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method and was analyzed by enzymatic digestion. The promoter sequence MPr1110 borne by the plasmid pMRT1110 was verified by sequencing (SEQ.ID07).

**3.3. Construction of the promoter MPr1111.**

Th promoter MPr1111 was created by inserting an 18 bp element containing a "G" box (stretching from positions -204 bp to \186 bp of the petE promoter, SEQ.ID01) at position -99 bp of MPr1098 (SEQ.ID04), and by fusing into this minimal promoter a sequence of 58 bp corresponding to a duplication of the as-2 element (Lam and Chua, 1989) and the as-1 element (Lam et al., 1989) of the CaMV 35S. MPr1111 (Fig. IV) was synthesized by the lb-PCR technique as described previously.

The single stranded continuous DNA was generated using the following "directional" oligodesoxynucleotides :

- S1 =

5' TTCCCTTCAAACACATACAAATTCAGTAGAGAAGAAACTCATTACTCTTGAGAAACCTAG

AGGATCCCCG 3' (SEQ.ID12)

- S2 =

5' CACAAAAACCCAATCCACATCTTTATCATCCATTCTATAAAAAATCACCTTCTGTGTGTC TCTCTTTCGA 3' (SEQ.ID13)

- S5 =

5' CTGTGGCACATCTACATTATCTAAATCTAAGCCACGTCGGAGGATAACATATTCTTCCAC ACATCTTAGCCA 3' (SEQ.ID16)

- S7 =

5' CATGCTGCAGACTAGTGATTGATGTGATATCAAGATTGATGTGATATCTCCACTGACGTA AGGGATGACGCATGCCACT 3' (SEQ.ID18)

One hundred picomole (100 pmol) of the S1, S2 and S5 oligodesoxynucleotides were phosphorylated in the 5' region through the use of 15 units of kinase (Amersham) in the presence of 5 µl of 10X kinase buffer (Amersham) and 500 pmol of ATP (Sigma), for 30 min at 37°C. Les phosphorylated oligodesoxynucleotides were purified by extraction with a volume of phenol, then a volume of phenol : chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally a volume of chloroform: isoamyl alcohol (24:1 v/v), before being precipitated by 1/10 volume 3M sodium acetate at pH 4.8 and 2.5 volumes of absolute ethanol at -80°C for 20 min then centrifuged at 16060 g for 30 min. The precipitated oligodesoxynucleotides were washed in 70% ethanol, dried, then resuspended in water at a concentration of 10 pmol/µl.

In order to link up the "directional" oligodesoxynucleotides, the following "guide" oligodesoxynucleotides were used :

- G1= 5' TGTGTTTGAAGGGAATCGAAAGAGAGACACA 3' (SEQ.ID19)
- G2= 5' GATTGGGTTTTTGTGTGGCTAAGATGTGTG 3' (SEQ.ID20)
- G4= 5' TGTAGATGTGCCACAGAGTGGCATGCGT 3' (SEQ.ID22)

In order to carry out the LCR reaction, 10 pmol of the phosphorylated S1, S2, S5 and S7 "directional" oligodesoxynucleotides were ligated in the presence of 10 pmol of the "guide" oligodesoxynucleotides G1, G2 and G4, 5 µl of Taq 10X DNA ligase buffer (New England Biolabs) and 40 units of Taq DNA ligase (New England Biolabs). The ligation reaction was carried out in a GeneAmp PCR System 9700 thermocycle (Perkin

Elmer, Norwalk, USA) and was comprised of one cycle for 1 min at 94°C, and then 8 identical cycles each comprising the following successive steps : 1 min at 65°C, 1 min at 57°C, 1 min at 52°C, 1 min at 48°C, 1 min at 43°C, and finally 10 min at 37°C. Next, the ligation reaction mixture was purified on a Qiaquick column according to the recommendations of the supplier.

Finally, the PCR amplification of the obtained single DNA strand was carried out in a GeneAmp PCR System 9700 thermocycle in the presence of 100 pmol each of the oligodesoxynucleotide probes 5' CATGCTGCAGACTAGTGGATT 3', and 5' CGGGGATCCTCTAGGTTTCT 3', 50 nmol of each of the dNTP, 10 µl of Vent 10X DNA polymérase buffer (New England Biolabs), and 2 units of Vent DNA polymérase (New England Biolabs). The DNA was denatured for 5 min at 94°C, subjected to 25 cycles each comprising a 30 sec denaturing step at 95°C, of a 30 sec hybridization step at 56°C, and of 1 min of elongation at 72°C, then continued elongation at 72°C for 5 min.

The DNA fragments of the reaction mixture were digested with 20 units of BamHI for 45 min at 37°C, then by 20 units of PstI for 1 h at 37°C, and finally purified on a Qiaquick column. They were inserted into the plasmid pGEM3Z-petE /IV2, which had been previously digested by BamHI enzyme for 1 h at 37°C and then by PstI enzyme for 1 h at 37°C, subjected to 0.8% agarose gel electrophoresis, purified on a Quiaquick affinity column, dephosphorylated for 1 h at 37°C in the presence of 12 µl of "Buffer 3" 10X (New England Biolabs) and of 5000 units of calf intestine alkaline phosphatase (CIP, New England Biolabs), and finally purified on a Quiaquick affinity column. To carry out the ligation, 25 ng of plasmid as treated above was contacted with 100 ng of the DNA fragments obtained by PCR, in the presence of 1.2 µl of T4 10X DNA ligase buffer (New England Biolabs) and 400 units T4 DNA ligase (New England Biolabs) for 1 night at 18°C. Previously prepared viable and competent Escherichia coli DH5α cells, were transformed with half of the ligation reaction mixture. The DNA from the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was

extracted according to the alkaline lysis method and was analyzed by enzymatic digestion. Two resulting plasmids, pMRT1111 and pMRT1112, were sequenced. The plasmid pMRT1111 contains the expected promoter Mpr1111 (SEQ.ID08), whereas in the plasmid pMRT1112, the promoter MPr1112 (Fig. IV) differs from MPr1111 by a deletion of 35 bp containing the "G" box and stretching from position -127 to position -89 and also a deletion of two bp situated in positions -78 and -76 (SEQ.ID09).

**3.4. Construction of the promoter MPr1153.**

The promoter MPr1153 (Fig. IV) was obtained by fusing the sequence of 78 bp from the petE promoter stretching from position -582 to position -510 bp (SEQ.ID01) and bearing the "as-1 like" and "nos enhancer like" elements into the promoter MPr1098 which was modified by the adjunction of the 18 bp element containing the "G" box.

To to this, the plasmid pMRT1111 was digested with 20 units of PstI enzyme and 1 unit of DraIII enzyme for 1 h at 37°C. The plasmid thus deleted of the 72 bp fragment containing the two "as-2" elements and the "as-1" element of CaMV, was isolated by 0.8% agarose gel electrophoresis, then purified on a Quiaquick affinity column. The 78 bp PstI/DraIII fragment containing the two "as-1 like" and "nos enhancer like" elements of the petE promoter was generated by digesting 10 µg of the plasmid pMRT1108 with 20 units of PstI enzyme and 1 unit of DraIII enzyme for 1 h at 37°C, then the fragment was isolated by Nu-Sieve 3% agarose gel electrophoresis (FMC, Rockland, USA) and finally purified on a Quiaquick affinity column.

The ligation was carried out with 20 ng of vector pMRT1111 PstI/DraIII and 80 ng of the 78 bp fragment for 1 night at 18°C in a reaction mixture of 10 µl in the presence of 1.0 µl of T4 10X DNA ligase buffer (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs). Previously prepared viable and competent Escherichia coli DH5α cells were transformed with hald of the ligation reaction mixture. The plasmid DNA from the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline

lysis method, and analyzed by enzymatic digestion. The plasmid obtained was designated pMRT1153 and the promoter sequence MPr1153 (SEQ.ID10) verified by sequencing.

**3.5. Construction of the promoter Mpr1143.**

The promoter MPr1143 (Fig. IV) was obtained by deleting the 72 bp sequence bearing the "as-2, as-2, as-1" elements of MPr1111. This was achieved by digesting the plasmid pMRT1111 for 1 h at 37°C simultaneously with 20 units of PstI enzyme and 1 unit of DraIII enzyme. The plasmid thus deleted of the 70 bp fragment containing the two as-2 elements and the as-1 element of CaMV were isolated by 0.8% agarose gel electrophoresis, then purified on a Quiaquick affinity column. The ends of this fragment were made blunt by the action of Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the supplier's recommendations. This fragment was religated for 1 night at 18°C in a 10 µl reaction mixture containing 20 ng of vector, 1.0 µl of the T4 10X DNA ligase buffer (New England Biolabs) and 400 units T4 DNA ligase (New England Biolabs). Previously prepared viable and competent Escherichia coli DH5α cells were transformed with half of the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method and analyzed by enzymatic digestion. The promoter sequence MPr1143 (SEQ. ID11) of one of these clones was verified by sequencing.

**Example 4.**

**Construction of binary plasmids containing the promoters MPr1151, MPr1149, MPr1170 and MPr1092.**

The preparation of the binary vector was the same for each of the expression cassettes containing MPr1111, MPr1098, Mpr1143 and MPr1092. A 25 µg amount of the plasmid pGA492 (An, 1986) was digested with 80 units of HindIII enzyme for 1 h at 37°C, then purified on a Quiaquick affinity column. The protruding 5' ends of this plasmid were blunted using Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the recommendations of the supplier. The thus modified plasmid was digested with 80 units of EcoRI enzyme for 1 h at 37°C, then the resulting vector

deleted of a 291 bp fragment was separated on 0.7 % agarose gel and purified on a Quiaquick affinity column.

**4.1. Production of pMRT1151.**

The expression cassette "MPr1111/uidA-IV2/nos term" was inserted at the modified HindIII site of the binary plasmid pGA492. It was obtained from the plasmid pMRT1111, previously digested with 80 units of PstI enzyme for 1 h at 37°C and purified on a Quiaquick affinity column. The protruding 5' ends of this plasmid were blunted using Pfu DNA polymérase (Stratagene, La Jolla, USA) according to the supplier's recommendations. The thus modified plasmid was digested with 80 units of EcoRI enzyme for 1 h at 37°C, then the 2.5 kb DNA fragment corresponding to the expression cassette was separated on 1 % agarose gel and purified on a Quiaquick affinity column.

The ligation was carried out by mixing 100 ng of binary plasmid pGA492 prepared as described above and 50 ng of expression cassette for 1 night at 18°C, in a 20 µl reaction volume in the presence of 2 µl of the T4 10X DNA ligase buffer (New England Biolabs) and 400 units T4 DNA ligase (New England Biolabs).

Previously prepared viable and competent Escherichia coli DH5α cells were transformed with half the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with tetracycline (12 mg/l), was extracted according to the alkaline lysis method and analyzed by enzymatic digestion as well as by gene amplification with the help of the oligodesoxynucleotides 5' ATATGAGACTCTAATTGGATACCGAGGGG 3', selected from the transfer DNA of the binary plasmid and 5' TTGATTTCACGGGTTGGG 3', selected from the expression cassette around the uidA sequence. The resulting clone was designated pMRT1151.

**4.2. Production of the binary plasmid pMRT1149.**

The expression cassette "MPr1143/uidA-IV2/nos term" was cloned at the modified HindIII site of the binary plasmid pGA492, following the same protocol as for plasmid pMRT1151, with the exception that the expression cassette was isolated from the plasmid pMRT1143. The resulting clone was designated pMRT1149.

**4.3. Production of the binary plasmid pMRT1170.**

The expression cassette "petE promoter/uidA-IV2/nos term" was cloned at the modified HindIII site the binary plasmid pGA492 , following the same protocol as for plasmid pMRT1151, with the exception that the expression cassette was isolated from the plasmid pGem3Z-petE/IV2

**4.4. Production of the binary plasmid pGA492MPr1092.**

The promoter fragments MPr1092 and sequence "uidA-IV2/nos term" were inserted into the binary plasmid pGA492 prepared as described above. The fragments were prepared in the following manner :

The CaMV D35S promoter was isolated by digesting 10 µg of the plasmid pJIT163Δ with 40 units of KpnI enzyme for 1 h at 37 °C. The ends of this linear plasmid were blunted with the help of 6 units of T4 DNA polymerase (New England Biolabs) for 30 min at 37°C according to the manufacturer's recommendations. The thus modified plasmid was purified on a Quiaquick affinity column, then redigested with 80 units of HindIII enzyme for 1 h at 37°C. The 743 bp fragment corresponding to the promoter was separated on 0.8 % agarose gel, then purified on a Quiaquick affinity column.

The "uidA-IV2/nos term" sequence was obtained by digesting 4 µg of the plasmid pMRT1092 with 40 units of HindIII enzyme and EcoRI enzyme for 1 h. The 2.2 kb fragment corresponding to the sequence "uidA-IV2/nos term" was separated on 0.8 % agarose gel, then purified on a Quiaquick affinity column.

The ligation between the three fragments was carried out by mixing 100 ng of binary plasmid, 50 ng of promoter fragment and 50 ng of the fragment corresponding to the sequence "uidA-IV2/nos term" in a reaction volume of 20 µl, in the presence of 2 µl of T4 10X DNA ligase buffer (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs). The incubation was carried out in a thermocycle by subjecting the ligation mixture to 198 cycles each comprising a 30 sec incubation at 30°C, and a 30 sec incubation at 10°C. Previously prepared viable and competent Escherichia coli DH5α cells were

transformed with half the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with tetracycline (12 mg/l), was extracted according to the alkaline lysis method and analyzed by enzymatic digestion and gene amplification with the help of the oligodesoxynucleotides 5' ATATGAGACTCTAATTGGATACCGAGGGG 3', selected from the transfer DNA of the binary plasmid and 5' TTGATTTCACGGGTTGGG 3', selected from the expression cassette in the "uidA" sequence. One of the clones retained was designated pGA492MPr1092.

**4.5 Production of the binary plasmid pMRT1182**

The binary plasmid pMRT1182 was obtained by insertion of the promoter fragment CaMV D35S and of the sequence uidA-IV2/term-nos in the binary plasmid pMRT1118. This latter plasmid is described completely in French patent application number FR 99 11112, filed on September 3, 1999, in the name of the present applicant, the specific description of which is incorporated herein by reference. The binary plasmid pMRT1118 (5971 pb) results from the introduction of a T-DNA fragment digested by AvrII enzyme into the AvrII site of another dephosphorylated plasmid also fully described in the previously mentioned prior application to same applicant, and designated pMRT1106, also specifically incorporated herein by reference. In order to carry out the insertion, the pMRT1106 plasmid DNA (5 µg) was digested with AvrII enzyme, purified with the aid of the « QIAquick PCR Purification » kit, then dephosphorylated with 50 units of calf intestine alkaline phosphatase (New England Biolabs) in a final reaction mixture volume of 120 µl in the presence of 12 µl 3x 10 buffer (New England Biolabs) at 37 °C for 1 hour, isolated by electrophoresis on à 0.6% agarose gel in TBE buffer, purified with a « QIAquick Gel Extraction » kit, dephosphorylated a second time with the calf intestine alkaline phosphatase under the conditions mentioned above, and finally purified with a « QIAquick PCR Purification » kit and transferred to 50 µl de $H_2O$.

The PCR ligation reaction was carried out with 32,5 ng of digested dephosphorylated plasmid pMRT1106 and 50 ng of T-DNA

fragments digested in a reaction mixture volume of 10 µl in the presence of 1 µl T4 10x DNA ligase buffer (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs). The ligation comprised 180 cycles each including 2 steps, the first one at 10°C for 30 seconds and the second step at 30°C for 30 seconds in a « GeneAmp PCR System 9700 » thermocycle.

Previously prepared viable and competent Escherichia coli DH5α bacteria, were transformed (Hanahan, 1983). The plasmid DNA of the obtained clones, selected on LB media supplemented with kanamycin (50 mg/l), was extracted according to the alkaline lysis method (Birnboim et Doly, 1979) and verified by enzymatic digestion and sequencing. The resulting plasmid was designated pMRT1118.

The promoter CaMV D35S was isolated by digesting 10 µg of plasmid pJIT163Δ successively with KpnI and HindIII enzymes for 1 hour at 37°C. The 743 bp fragment corresponding to CaMV D35S was separated on 0.8% gel agarose, and then purified on a Qiaquick affinity column. The sequence "uidA-IV2/nos term" was obtained by digesting the plasmid pMRT1092 with 40 units of HindIII and EcoRI enzymes for 1 hour. The 2.2 kb fragment corresponding to the required sequence was separated on 0.8% gel agarose, then purified on a Qiaquick affinity column. In parallel, 10 µg of binary plasmid pMRT1118 were digested successively with KpnI and EcoRI enzymes for 1 hour at 37°C. The linearized vector fragment was then dephosphorylated with 40 units of calf intestine alkaline phosphatase (New England Biolabs) in the presence of 3X buffer for 1 hour at 37°C. The ligation was carried out in the presence of 100 ng of binary plasmid, 50 ng of the CaMV D35S fragment and 50 ng of the fragment corresponding to "uidA-IV2/nos term" in a reaction volume of 20 µl, in the presence of T4 (1X) DNA ligase buffer and 400 units of T4 DNA ligase (New England Biolabs). Incubation was carried out by PCR cycles in a "GeneAmp PCR System 9700" thermocycle as described previously. Previously prepared viable and competent Escherichia coli DH5α bacteria were transformed with half of the ligation reaction mixture. The plasmid DNA of

the obtained clones, selected on LB media supplemented with kanamycin (50 mg/l), was extracted according to the alkaline lysis method and analysed by enzymatic digestion. The resulting plasmid was designated pMRT1182.

The plasmids pMRT1151, pMRT1149, pMRT1170 and pMRT1182 were transferred into the strain Agrobacterium tumefaciens LBA4404 according to the technique described by Holsters et al. (1978). The plasmid DNA of the obtained clones, selected on LB media supplemented with rifampicine (50 mg/l) and tetracycline (5 mg/l), was extracted according to the alkaline lysis method, and modified by adding lysozyme (25 mg/ml) to the cell resuspension buffer. The plasmid DNA obtained was analyzed by enzymatic digestion and by gene amplification with the help of the oligodesoxynucleotides 5' ATATGAGACTCTAATTGGATACCGAGGGG 3' selected from the transfer DNA of the binary plasmid and 5' TTGATTTCACGGGTTGGG 3' selected from the expression cassette around the "uidA" sequence. The Agrobacterium clones obtained were used to carry out Agrobacterium mediated plant genetic transformation.

**Example 5.**

**Measure and comparison of the expression levels of the different promoters using transient expression techniques.**

**5.1 In vitro culture of tobacco, leaf preparation.**

The transient expression experiments were carried out on tobacco leaves (Nicotiana tabacum L.) of the cultivar bpD6 aged 6 weeks. Mature seeds of tobacco cv. bpD6 were sterilised for 10 min in a saturated calcium hypochlorite solution (70 g/l), then rinced three times for 5 min in sterile deionized water. The sterile seeds were then placed on MS20 media (Murashige and Skoog, 1962) and incubated for 6 weeks in a culture chamber (constant temperature of 24°C, photoperiode 16 h obscurity / 8 h light, light intensity of 200 µmol photons.m-2.sec-1).

In order to avoid splitting of the foliar mesophyll cells during transformation, the major leaves of the bpD6 tobacco plants aged 6 weeks were excised from the plant 24 h before gene gun transformation, and placed, lignous face up, on light

plasmolysis BY3 media (MS Salts 4,4 g/l, myoinositol 100 mg/l, thiamine 1 mg/l, $KH_2PO_4$ 200 mg/l, Saccharose 30 g/l, Sorbitol 45,5 g/l, 2,4 D 1 mg/l, pH 5,8).

**5.2. Gold Particle Coating with DNA of the Chimeric Constructions.**

Gene gun transformation requires prior coating of DNA onto gold bead spheres of 0,6 mm in diameter, that have been sterilized for 10 min absolute ethanol (99,98 %, at less than 0,02 % water), washed four times in sterile deionized water, and finally preserved for a maximum of 4 weeks maximum at -20°C in a 50% glycerol solution.

The concentration of all of the control and experimental plamsids used during transformation was adjusted to 1 mg/ml. In each transformation experiment, an internal reference control (pCaMV35Sluc) was cotransformed in order to normalize the variations in GUS activity between the different experiments (Leckie and al., 1994).

The coating of DNA onto the previously prepared gold beads was carried out in a sterile container under laminar flow conditions. An aliquot of 1,8 mg of sterile beads in suspension in 30 µl of 50 % glycerol, was vigourously mixed in a vortexer for 1 min, then for 10 sec with 20 µl of DNA suspension containing 4 µg of one of the plamsids to be tested and 2 µg of the reference plasmid pCaMV35Sluc. Then, 20 µl of $CaCl_2$ 2,5 M were added and mixed vigourously for 10 sec. Next, 20 µl of spermidine 0,1 M were added to the mixture and whole mixture was agitated under vortex for 30 further seconds. The coating of DNA onto the beads was continued by incubating the mixture in ice for 15 min, then the coated beads were centrifuged at low velocity for 5 sec and washed twice in absolute ethanol.

After washing, the coated beads were resuspended in 32 µl of absolute ethanol, subjected to ultrasound three times each for 2 sec, vigourously mixed in the vortexer for 15 sec, then immediately split into 4 identical equal aliqouts on sterile macrocarrier disks used in a Biolistic PDS-1000/He system prepared according to the manufacturer's recommendations

(Bio-Rad, Hercule, USA). The whole arrangement of macrocarrier support and macrocarrier bearing the deposited beads was left to dry for 5 min.

**5.3. Bombardment of foliar tissues of tobacco and transient expression.**

The bombardment of tobacco leaves was carried out with the help of a Biolistic PDS-1000/He system following the general manufacturer's recommendations (Bio-Rad, Hercule, USA) with respect to handling and mounting of the different components of the apparatus. Each leaf was bombarded twice succesively using the following conditions :

- helium pressure used to accelerate the beads was equal to 6200 kPa (900 psi).
- the plant sample was placed at a distance of 9 cm from the bead acceleration zone.
- the bombardment was carried out in a vacuum of 27 mm of mercury.

After bombardment the leaves were left in BY3 medium and incubated for 48 h in the dark in a culture chamber at 24°C. This incubation enables transient expression of the trangenes introduced into the cells.

**5.4. Evaluation of the activity of different promoters using histochemical staining.**

The revelation of the expression of ß-glucuronidase was carried out by histochemical staining as described by Jeffersson et al. (1987). After 48 h in the culture chamber, each leaf was cut into two along the axis of the central spine. Half of the leaf was incubated in ß-glucuronidase staining buffer (5-bromo, 4-chloro, 3-indolyl glucuronide (X-Gluc) 500 mg/l, Triton x100 0.05% in 0.1 M, pH 7.0 phosphate buffer) for 48 h at 37°C, whrereas the other half was frozen in liquid nitrogen, then conserved at -80°C.

After staining, the leaves were bleached by dipping them into two 95 % ethanol baths for respectively 3 and 12 h, then rinsed in distilled water and dried flat between two sheets of cellophane.

The promoter activity of the different constructs was evaluated by the number of blue spots revealed on each leaf after two bombardments amounting to 2 µg of DNA bearing the GUS reporter gene.

Three categories of promoters were identified. The leaves bombarded with the promoters MPr1096, MPr1098, MPr1108, MPr1109, MPr1143 and MPr1153 all showed an average of less than 30 blue spots. The leaves bombarded with the promoters petE, MPr1097 and MPr1110 all showed a number of blue spots between 50 and 150. Finally , the leaves bombarded with MPr1111 and the reference promoter MPr1092 had a very large number of blue spots on average and in general greater than 200.

In conclusion, the chimeric promoters MPr1110 and MPr1111 enable expression of permettent d'obtenir une expression of ß-glucuronidase at a level greater than or equal to that of the whole petE promoter ; and MPr1111 shows promoter activity at least as comparable as that obtained by the strong constitutive reference promoter D35S prom.

**5.5. Quantification of the expression of ß-glucuronidase by the different promoters using luminometric enzyme assay.**

The frozen leaf halves were ground in a mortar, and the powder was left to defrost in the extraction buffer at 1 ml of buffer for 200 mg of plant tissue (Tris Phosphate 25 mM pH 7.8, Dithiothreitol 2 mM, 1,2-diaminocyclohexane N,N,N',N'-tetracetic acid 2 mM, glycerol 10 %, Triton x100 1 %). The mixture was homogenized then incubated for 15 min in ice before being clarified by centrifugation for 5 min at 16060 g.

The GUS activity was measured on 20 µl crude clarified leaf extract with the help of the detection kit "GUS-Light chemiluminescent reporter gene assay" (Tropix Inc., Bedford, USA) according to the supplier's recommendations. The measurement of light emission was carried out with the help of a Lumat LB 9507 luminometer (EGG-Berthold, Bad Wildbad, Allemagne).

Luciferase activity was measured on 20 µl crude leaf extract

with the help of the detection kit "Luciferase assay system" (Promega Corp., Madison, USA) according to the supplier's recommendations. Light emission measurement was effected with the help of a Lumat LB 9507 luminometer.

The results are reported in Fig. V. For each experiment (one bombarded leaf = one crude extract), the ratio between the measured ß-glucuronidase activity and luciferase activity as measure by the luminometer, was calculated. The average of the different experiments for a given construct and mean standard error were determined.

The promoters can be divided into 6 classes ordered on an increasing scale of expression starting from the weakest (class 1) to the strongest (class 6) :

- Class 1 inclues the promoters MPr1108 and MPr1109 (Fig. IV). The expression conferred by these promoters seems to differ very little from that obtained with the construction pMRT1144 (Fig. I), which contains no promoter. The fusion of the "as-1 like" and "nosE like" boxes to the minimal promoter MPr1098 (Fig. IV) to obtain MPr1108, only slightly lowers the average expression promoted by MPr1098. This result seems to suggest that a very slight inhibitor effect is due to these boxes and their position within MPr1108. The promoter MPr1109 confers an essentially identical expression to that obtained with promoter MPr1108. The deletion of the sequence siutated downstream of the transcription initiation site, that is to say the 5' untranslated region, does not appear to modify the expression obtained with MPr1108.
- Class 2 comprises promoters MPr1098, MPr1143 and MPr1112 (Fig. IV). The promoters MPr1098 and MPr1143 show similar activity. The promoter Mpr1143 results from the insertion of a "G" box at a distance of 36 bp upstream of the "CAAT" box in the minimal promoter MPr1098. The presence of this box does not appear to have a significant effect on the expression obtained with the promoter MPr1098. The promoter MPr1112 includes upstream of the "CAAT" box, a duplication of the "as-2" box followed by the "as-1" box originating from the promoter 35S of CaMV. These

elements on their own do not appear to contribute to improving the epxression rate which is essentially identical to that obtained with the promoter MPr1098.

- Class 3 includes promoters MPr1096 and MPr1097 (Fig. IV). The expression conferred by these two promoters is identical. The creation of MPr1096 by deletion of the fragments SphI-SpeI bearing the "nosE like" box and SpeI-SpeI of MPr1097, bearing a region from the sequence enhancer of the petE promoter (Fig. IV), does not appear to affect the expression rate. In promoter MPr1096, the presence of the "G" box, preceded by the 31 bp of the petE sequence enhancer and at a distance of 122 bp from the "CAAT" box appears to enable the increase in the expression rate obtained by MPr1098 by a factor of 2.5. It should be noted that in the case of MPr1143, the présence of the "G" box preceded by the 28 bp petE sequence enhancer at a distance of 36 bp from the "CAAT" box does not permit an increase in the rate of expression obtained with MPr1098. It would thus seem that the distance between the "G" and "CAAT" boxes influences the rate of expression.

- Class 4 includes promoter petE (Fig. IV) which is used as a reference.

- Class 5 comprises the promoter MPr1110 (Fig. IV). The fusion of the as-2 and as-1 boxes upstream of the promoter MPr1143 creating the promoter MPr1110 enables a considerable increase in the expression rate that is very much higher than that obtained with the petE promoter (by a factor of 1.4). It appears that the "G", "as-1" and "as-2" boxes together have an active positive synergic effect with respect to the expression.

- Class 6 covers promoters MPr1111 (Fig. IV) and MPr1092 (Fig. I). The promoter MPr1111 confers an expression rate similar to that of the reference double 35S CaMV promoter (MPr1092). The addition of an "as-2" element or box considerably increases the expression rate with respect to that obtained by MPr1110 (by a factor of 1.7). These elements appear to act synergistically. The duplication of the "as-2" box also increases this effect.

In conclusion, the chimeric promoter MPr1110 enables an average

expression of ß-glucuronidase at a level greater than or equal to the whole petE promoter. The promoter MPr1111 has a promoter activity comparable to the average activity obtained with the reference promoter D35S.

These results are in agreement with those observed after histochemical staining of the bombarded leaves with the same promoters.

The CaMV D35S promoter is commonly reported in the literature as being a strong promoter. The chimeric promoters of the present invention bring an increase in the promoter activity of the GUS reporter gene of the order of 8 to 12 times that of the reference promoter CaMV 35S (Kay and al., 1987).Furthermore, MPr1111 constitutes one of the most active and strongest chimeric promoters in tobacco leaves described to date.

The promoters of lesser strength can be used as promoters associated with genes coding for selection agents, for example, in order to confer antibiotic resistance, for example in the same way as promoters of the "nos" type.

**Example 6.**

**Expression of the different promoters in tobacco after stable transformation.**

**6.1. Stable transformation in tobacco.**

The transformation of tobacco (Nicotiana tabacum L., cultivar bpD6) was carried out by infecting foliar disks isolated from tobacoo plants aged 6 weeks by recombinant Agrobacterium according to the method described by Horsch et al. (1985). During transformation, the Petri dishes were incubated in a culture chamber under the following conditions : temperature of 24°C, photoperiod of 8 h darkness / 16 h light, luminous intensity of 200 µmol photons.m-2.sec-1 , and apart from the initial coculture step, all of the callogenesis, regeneration, and rooting steps were carried out on varying selective media supplemented with Augmentin ® (400 mg/l) and Kanamycin ® (200 or 100 mg/l) ;

The different steps and the media were the following :

- a coculture step lasting three, during which the Agrobacteria

infect the plant cells, on a solid MS30 coculture media (media based on MS (Murashige and Skoog, 1962) supplemented with vitamins (Gamborg and al., 1968) 4.4 g/l (Sigma, M0404), Saccharose 30 g/l, agar 8 g/l (Merck), pH 5.7), Benzyl Amino Purine at 1 mg/l and Indole-3 acetic acid at 0,1 mg/l .

- a bud tip formation step consisting of four weeks in a culture chamber on a solid MS20 regeneration media (Salts and vitamins MS 4,4 g/l (Sigma, M0404), Saccharose 20 g/l, , agar 8 g/l (Merck), pH 5.7), supplemented with Benzyl Amino Purine at 1 mg/l, Indole-3 Acetic acid at 0.1 mg/l, Augmentin ® at 400 mg/l and Kanamycine ® at 200 mg/l.
- a development and rooting step lasting three weeks in a culture chamber on MS20 solid development media supplemented with Augmentin ® at 400 mg/l and Kanamycine ® at 100 mg/l.
- a repotting step into glass pots in a culture chamber on sur MS20 solid development media supplemented with Augmentin ® at 400 mg/l and Kanamycine ® at 100 mg/l.

**6.2. Comparison of chimeric promoter activity after stable expression in tobacco plants.**

β-glucuronidase activity was measured in primary transformants 2, 4, 6, 8 and 10 weeks after their transfer to greenhouse. For each plant, 3 samples were taken and pooled together in a test tube. One sample was taken from an "old" leaf (basal foliar level), one from a mature leaf (of a median leaf level), and one on a young leaf located at the apex of the plant.

Each sample was ground with liquid nitrogen in a mortar and the powder was resuspended in extraction buffer (Tris phosphate 25 mM, pH=7.8, dithiothreitol 2 mM, 1,2-diaminocyclohexan, N, N, N',N'-tetracetic acid 2 mM, glycerol 10 %, Triton X100 1 %) at a ratio of 1 ml buffer per 200 mg plant powder. The material was homogenized, incubated for 15 min on ice before being clarified by centrifugation at 16060 g for 5 min.

GUS activity was measured on 20 ml of clarified crude extract with the help of the "GUS-Light chemiluminescent reporter gene assay" detection kit (Tropix Inc., Bedford, USA) according to the manufacturer's recommendations. Measurements of light

emission were carried out using the Lumat LB 9507 luminometer (EGG-Berthold, Bad Wildbad, Germany).

Total protein content in crude extract was assessed by the Bradford's technique (Bradford, 1976), using the "Bio-Rad protein assay" reagent (Bio-Rad, München, Allemagne) according to the manufacturer's recommendations.

Reporter gene activity in the different categories of plants was analyzed over the population of 20 transformants per construct, and over the whole period of plant growth and development. Analysis was not performed at the individual level because of random insertion sites and variable copy number in the different transformants of a given category.

The results for the chimeric promoter MPr1111, compared to the original p*PetE* promoter, the minimal MPr1143 and the reference MPr1092 promoter are presented Fig.VI.

GUS activity in MPr1092 reference plants showed relatively little variation compared to what was observed in plants transformed with plastocyanin-based promoters. Activity decreased slightly between 2 and 4 weeks after transfer of the plants to greenhouse; increased about 4 times at 6 weeks, dropped again at 8 weeks to values observed at 4 weeks and finally increased slightly at 10 weeks. Analysis of plastocyanin-based promoters revealed that whatever promoter or plant considered, GUS activity increased regularly from 2 to 6 weeks after transfer to greenhouse and decreased thereafter till flowering.

This evolution of GUS activity was correlated to plant developement, since the plastocyanin gene is actively expressed in photosynthetic tissues. Activities of p*PetE* and derived promoter therefore followed active growth of the plants spanning from greenhouse transfer to 6-8 weeks after acclimation, followed by a slower development of the plants from 8 weeks to flowering that occured 10-12 weeks after transfer to greenhouse. In comparison, the reference MPr1092 promoter, known as a highly active constitutive promoter was less dependent on these developmental associated effects.

Comparison of transformants bearing the chimeric promoter MPr1143 and the reference MPr1092 showed that whatever stage of development, "MPr1143 plants" exhibited a very low GUS activity compared to "D35S plants". These data revealed that MPr1143 was only able to drive minimal expression of the reporter gene at a basal level and confirmed the results obtained after transient expression experiments showing that the "G box" by itself, inserted into the minimal p*PetE* sequence was not sufficient to promote expression effectively.

Comparison of the population of transformants bearing the original p*PetE* promoter to the reference "MPr1092 plants" showed that p*PetE* was as active as the reference at 2 weeks after transfer of the plants to greenhouse, 3 to 4 times more active during active development (from 2 to 8 weeks). The differences between the two promoters decreased regularly from 6 weeks after transfer and was reversed at 10 weeks since reference plants exhibited an average activity greater than twice that of "p*PetE* plants". These data did not corroborate those obtained after transient expression, in which p*PetE* was 2,5 times less active than MPr1092.

Comparison between "MPr1111 plants" and those bearing p*PetE* revealed that MPr1111 was on average 2 to 3 times less active than p*PetE* till 6 weeks and was at least as active thereafter. At 8 and 10 weeks a few "MPr1111 individuals" were indeed more active than "*PetE* ones". To this extent, stable expression led to a different conclusion compared to transient expression in which MPr1111 was on average 2,5 more active than p*PetE*. MPr1111 was shown to be more active than the reference MPr1092 in the population of primary transformants over a period of growth and development extending form 4 to 8 weeks after transfer of plants to greenhouse, with a maximum of 2 to 3 times more activity at 8 weeks.

On the basis of these time course analyses we can conclude that respective strength of the promoters is not static throughout growth of the plants, and this is probably due to different regulation processes. MPr1111 is the promoter that drove

expression of the reporter protein GUS at the highest level 8 weeks after transfer of the plants to greenhouse, and therefore appears to be the best candidate to be used for expression of large quantities of heterologous proteins at this stage of tobacco development.

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5

SEQUENCE LISTING

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<303> Plant Journal
<304> 3
<306> 437-449
<307> 1993
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<303> Plant Journal
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<303> Plant Mol. Biol.

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<303> Plant Journal

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<307> 1993

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tgagaaacct agaggatcc 259

[CLAIMS]

1) Chimeric expression promoter comprising at least one nucleic acid sequence derived from a promoter of the pea plastocyanin gene having the sequence identified under the number SEQ.ID01.

2) Chimeric promoter according to claim 1, wherein the nucleic acid sequence derived from the promoter of the pea plastocyanin gene is selected from the group consisting of the sequences identified under the numbers SEQ.ID02, SEQ.ID03, SEQ.ID04, SEQ.ID05, SEQ.ID06, SEQ.ID07, SEQ.ID08, SEQ.ID09, SEQ.ID10, and SEQ.ID11.

3) Chimeric expression promoter comprising a "G" box operably or functionally linked upstream of at least one "CAAT" box, "TATA" box and transcription initiation site (+1).

4) Chimeric expression promoter according to claim 3, wherein the "G" box is situated between positions - 225 and - 65 with respect to the transcription initiation site (+1).

5) Chimeric expression promoter according to claim 3, wherein the "G" box is situated between positions - 201 and - 115 with respect to the transcription initiation site (+1).

6) Chimeric expression promoter according to claim 3, wherein the "G" box is situated at position - 201 with respect to the transcription initiation site (+1).

7) Chimeric expression promoter according to claim 3, wherein the "G" box is situated at position - 115 with respect to the transcription initiation site (+1).

8) Chimeric promoter according to claim 3, wherein the "G" box is of plant origin.

9) Chimeric promoter according to claim 3, wherein the "G" box is obtained from a promoter of the pea plastocyanin gene.

10) Chimeric promoter according to claim 3, wherein the "G" box is obtained from the petE promoter of the pea plastocyanin gene .

11) Chimeric promoter according to any one of claims 3 to 10, wherein it further comprises a "nos E like" box operably or

functionally linked upstream of the "G" box.

**12)** Chimeric promoter according to any one of preceding claims 3 to 11, wherein it further comprises at least one "as1" or "as1 like" box operably or functionally linked to the "G" box.

**13)** Chimeric promoter according to claim 12, wherein the promoter comprises two or more "as1" or "as1 like" boxes, arranged either contiguously or separately

**14)** Chimeric promoter according to claim 12 or claim 13, wherein the promoter comprises four "as1" or "as1 like" boxes.

**15)** Chimeric promoter according to any one of preceding claims 12 to 14, wherein the "as1" or "as1 like" boxes are linked both upstream and downstream of said "G" box., and preferably are linked upstream thereof.

**16)** Chimeric promoter according to any one of preceding claims 12 to 15, wherein one or more of the the "as1" or "as1 like" boxes is arranged in inverse order, and preferably in inverse repeat order.

**17)** Chimeric promoter according to any one of the preceding claims 3 to 16, wherein it further comprises at least one "as2" box operably linked to the "G" box.

**18)** Chimeric promoter according to any one of the preceding claims wherein the promoter comprises at least two or more "as2" boxes, and preferably four "as2" boxes.

**19)** Chimeric promoter according to claim 18, wherein said "as2" boxes are linked both upstream and downstream of said "G" box, and preferably are linked upstream thereof.

**20)** Chimeric promoter according to any one of preceding claims 17 to 19, wherein one or more of the the "as1" or "as1 like" boxes is arranged in inverse order, and preferably in inverse repeat order.

**21)** Chimeric promoter according to any one of the preceding claims 3 to 20, wherein it comprises at least one nucleic acid sequence selected from the group consisting of the sequences identified under the numbers SEQ.ID02, SEQ.ID03, SEQ.ID04, SEQ.ID05, SEQ.ID06, SEQ.ID07, SEQ.ID08, SEQ.ID09,

SEQ.ID10, SEQ.ID11.

**22)** Expression cassette comprising at least one nucleic acid sequence derived from a promoter of the pea plastocyanin gene, operably or functionally linked to a nucleic acid sequence to be expressed coding for a polypeptide to be produced, itself operably or functionally linked to a transcription termination nucleic acid sequence, wherein the nucleic acid sequence derived from a promoter of the pea plastocyanin gene is selected from the group consisting of the sequences identified under the numbers SEQ.ID02, SEQ.ID03, SEQ.ID04, SEQ.ID05, SEQ.ID06, SEQ.ID07, SEQ.ID08, SEQ.ID09, SEQ.ID10, and SEQ.ID11.

**23)** Isolated promoter nucleic acid sequence, wherein the sequence is selected from the group consisting of the sequences identified under the numbers SEQ.ID02, SEQ.ID03, SEQ.ID04, SEQ.ID05, SEQ.ID06, SEQ.ID07, SEQ.ID08, SEQ.ID09, SEQ.ID10, and SEQ.ID11.

**24)** Directional desoxynucleotide building block for a chimeric expression promoter or an isolated promoter nucleic acid sequence according to any one of claims 1 to 21 or 23, wherein the sequence is selected from the group consisting of the sequences identified under the numbers SEQ.ID12, SEQ.ID13, SEQ.ID14, SEQ.ID15, SEQ.ID16, SEQ.ID17 and SEQ.ID18.

**25)** Guide desoxynucleotide building block for a chimeric expression promoter or an isolated promoter nucleic acid sequence according to any one of claims 1 to 21, or 23, wherein the sequence is selected from the group consisting of the sequences identified under the numbers SEQ.ID19, SEQ.ID20, SEQ.ID21 and SEQ.ID22.

**26)** Vector comprising a promoter, or a promoter nucleic acid sequence, capable of initiating transcription of nucleic acid sequence coding for a polypeptide to be produced, wherein the promoter or the promoter nucleic acid sequence correspond to a chimeric expression promoter or a promoter nucleic acid sequence according to any one of claims 1 to 21, or 23.

**27)** Vector according to claim 26, wherein the vector is chosen from the group consisting of the binary vectors

idenditified under the numbers pMRT1151, pMRT1149, pMRT1170.

**28)** Method for the manufacture of a chimeric expression promoter or an isolated promoter nucleic acid sequence according to any one of claims 1 to 21 or 23, wherein it comprises the steps consisting of :

- carrying out a ligation chain reaction, called LCR, that produces a continuous single stranded DNA from at least one directional desoxynucleotide building block selected from the group consisting of the "directional" desoxynucleotides S1, S2, S3, S4, S5, S6, and S7 identified under the numbers SEQ.ID12, SEQ.ID13, SEQ.ID14, SEQ.ID15, SEQ.ID16, SEQ.ID17 and SEQ.ID18 respectively, and at least one "guide" desoxynucleotide building block, for said promoter nucleic acid sequence or promoter, selected from the group consisting of the "guide" desoxynucleotides G1, G2, G3, and G4 identified under the numbers SEQ.ID19, SEQ.ID20, SEQ.ID21 and SEQ.ID22 respectively ;
- carrying out PCR amplification on the single stranded DNA obtained in the previous step enabling the production of a double stranded DNA corresponding to the chimeric expression promoter or the promoter nucleic acid ;
- optionally isolating the promoter or the promoter nucleic acid sequence.

**29)** Method according to claim 28, wherein the desoxynucleotide blocks are phosphorylated before ligation.

**30)** Method according to claim 28, wherein the ligation is carried out in the presence of at least one DNA ligase in a thermocycle, under the following conditions :

- one cycle of about one minute at about 94°C ;
- eight identical cycles each of which consists of the following steps :
- one minute at 65°C, one minute at 57°C, one minute at 52°C, one minute at 48°C, one minute at 43°C and ten minutes at 37°C.

**31)** Transgenic plant having stably integrated into its genome at least one promoter or at least one promoter nucleic acid sequence according to any one of claims 1 to 21, or 23

respectively.

**32)** Transgenic plant according to claim 31, wherein the plant is selected from dicotyledonous species, preferably potato, tobacco, cotton, lettuce, tomato, melon, cucumber, pea, rape, beetroot, or sunflower, or monocotyledonous species, preferably wheat, barley, oat, rice, or corn.

**33)** Propagule of a transgenic plant according to any one of claims 31 or 32.

**34)** Propagule of a trangenic plant according to claim 33, wherein the propagule is a seed.

**35)** Cell containing a promoter or a promoter nucleic acid sequence according to any one of claims 1 to 21, or 23 respectively.

**36)** Cell according to claim 35, wherein the cell is selected from plant cells, human cells, animal cells, insect cells, bacterial cells, algal cells, and fungal cells, and preferably is a plant cell.

**37)** Method for expressing a nucleic acid sequence, or gene, coding for a polypeptide to be produced, in a cell, wherein said method comprises the steps consisting of :

- transforming the cell with a vector comprising at least one promoter or at least one promoter nucleic acid according to any one of claims 1 to 21, or 23 operably linked to a nucleic acid sequence, or gene, coding for a polypeptide to be produced, itself operably linked to a transcription terminator signal ;

- culturing the transformed cell under conditions enabling the expression of the nucleic acid sequence, or gene, coding for the polypeptide, whereby the polypeptide is produced.

**38)** Method according to claim 37, wherein the cell is prokaryote or eukaryote cell.

**39)** Method according to any one of claims 37 or 38, wherein the cell is one chosen from the group consisting of microbial cells, fungal cells, insect cells, animal cells, and plant cells.

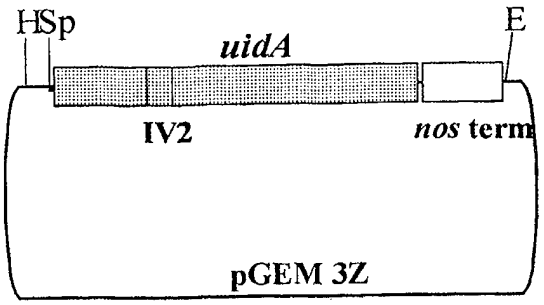
**40)** Method according to any one of claims 37 to 39,

wherein the cell is a plant cell.

**41)** Method for the manufacture of a transgenic plant according to any one of claims 31 or 32, or of a propagule according to claim 33, wherein the method comprises the steps consisting of :

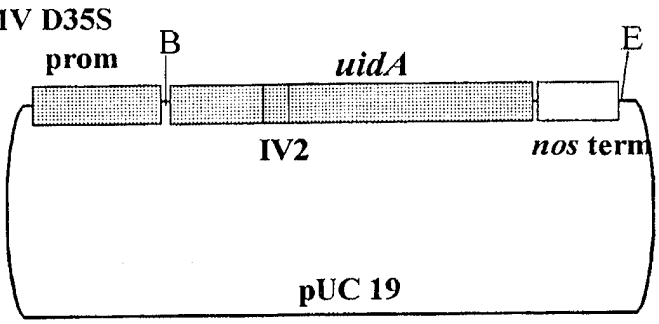
- transforming a plant cell with a vector comprising at least one promoter or at least one promoter nucleic acid sequence according to any one of claims 1 to 21, or 23 ;
- selecting the plant cell having integrated the promoter or the promoter nucleic acid sequence ;
- propagating the transformed and selected plant cell, either through culture, or through regeneration of whole chimeric or transgenic plants.

Fig. I




pMRT1144

Fig. II




pMRT1092

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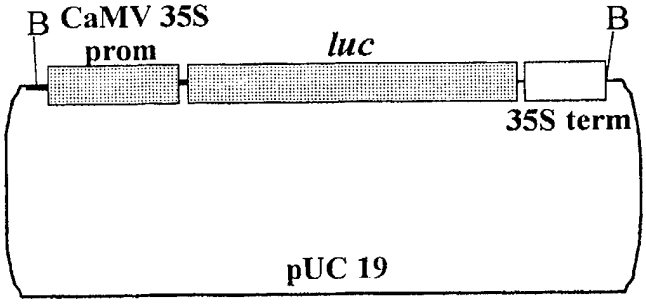


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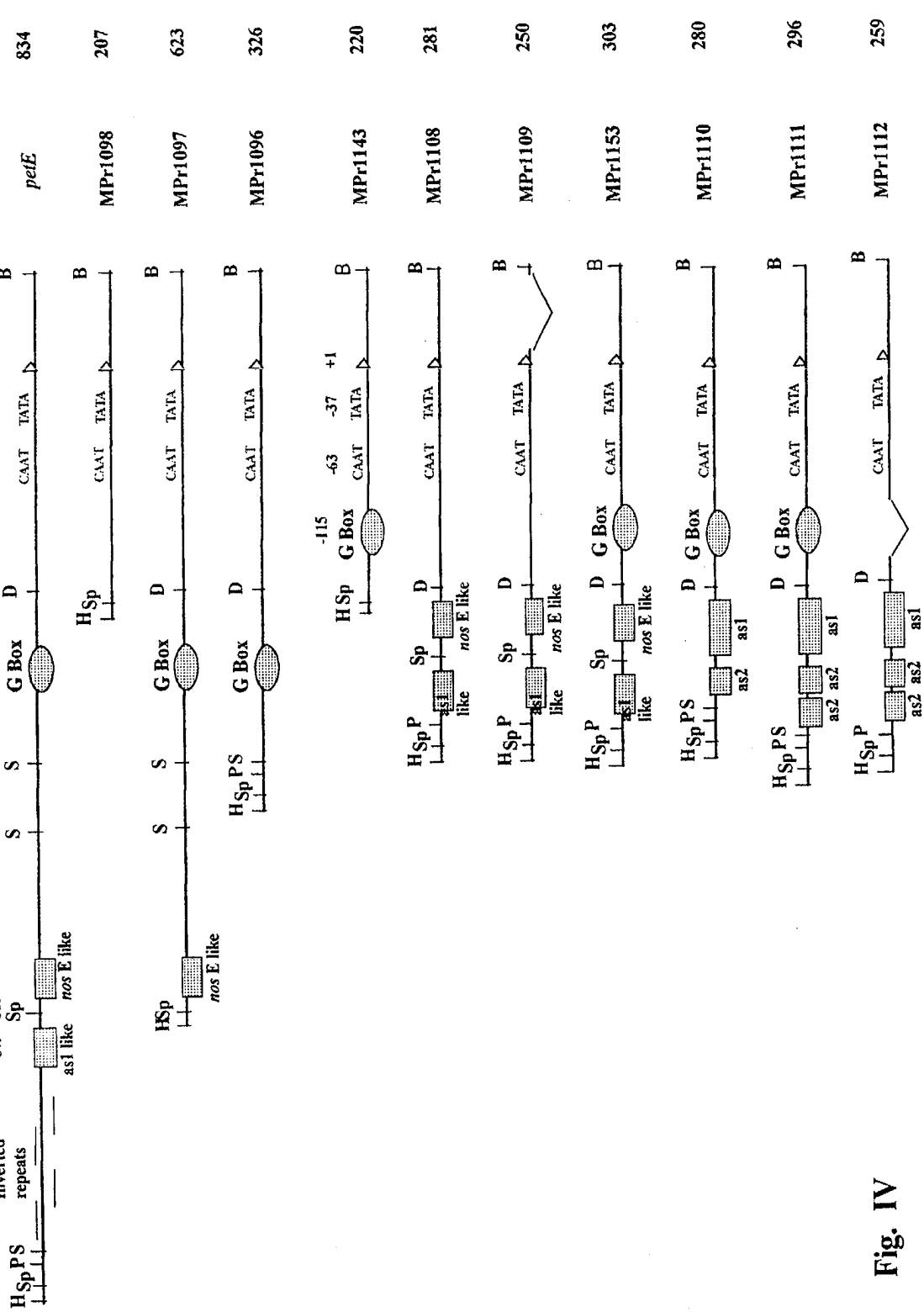
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623
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281
MPr1109
250
MPr1153
303
MPr1110
280
MPr1111
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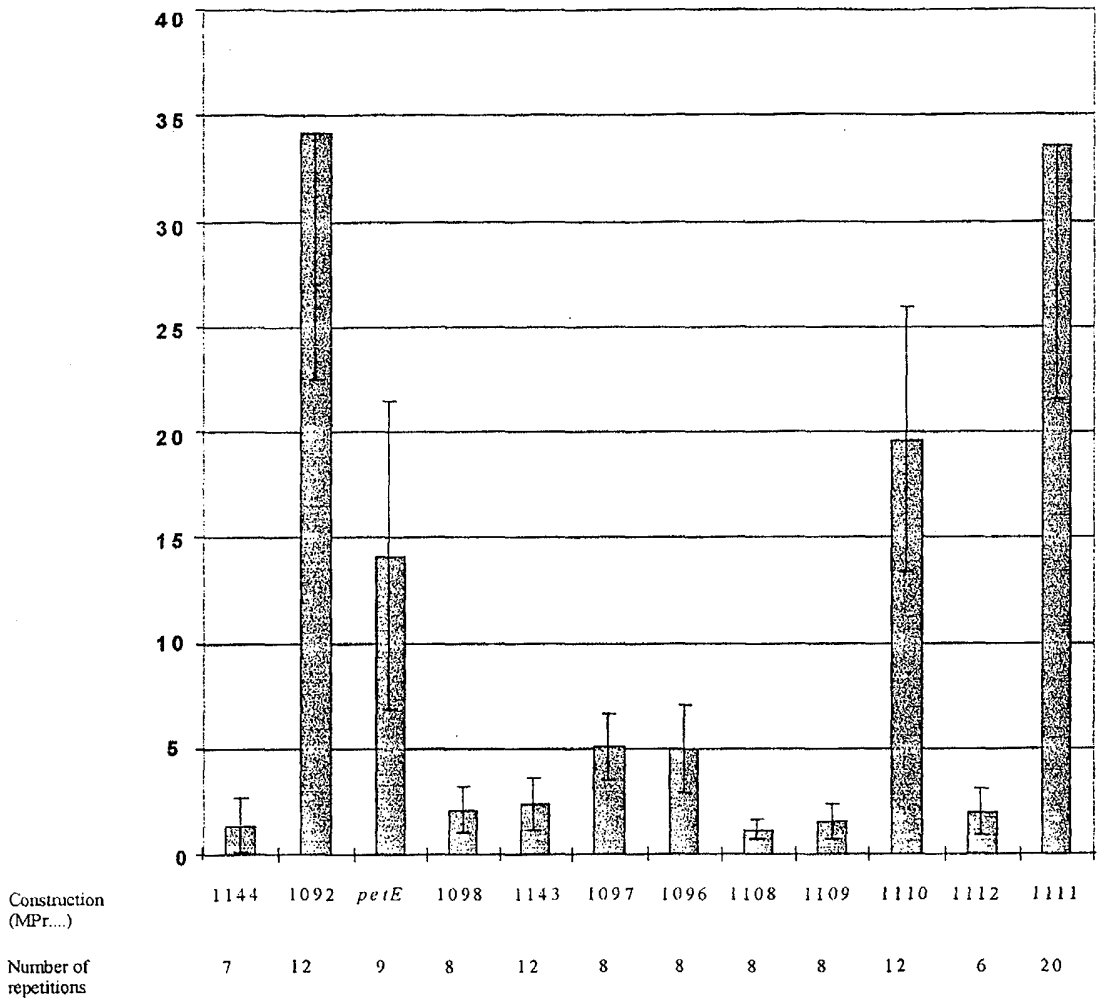
259
Inverted repeats
as1 like
nos E like
G Box
CAAT
TATA
HSp PS
HSp P
HSp

Fig. IV


40
35
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25
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10
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Number of repetitions
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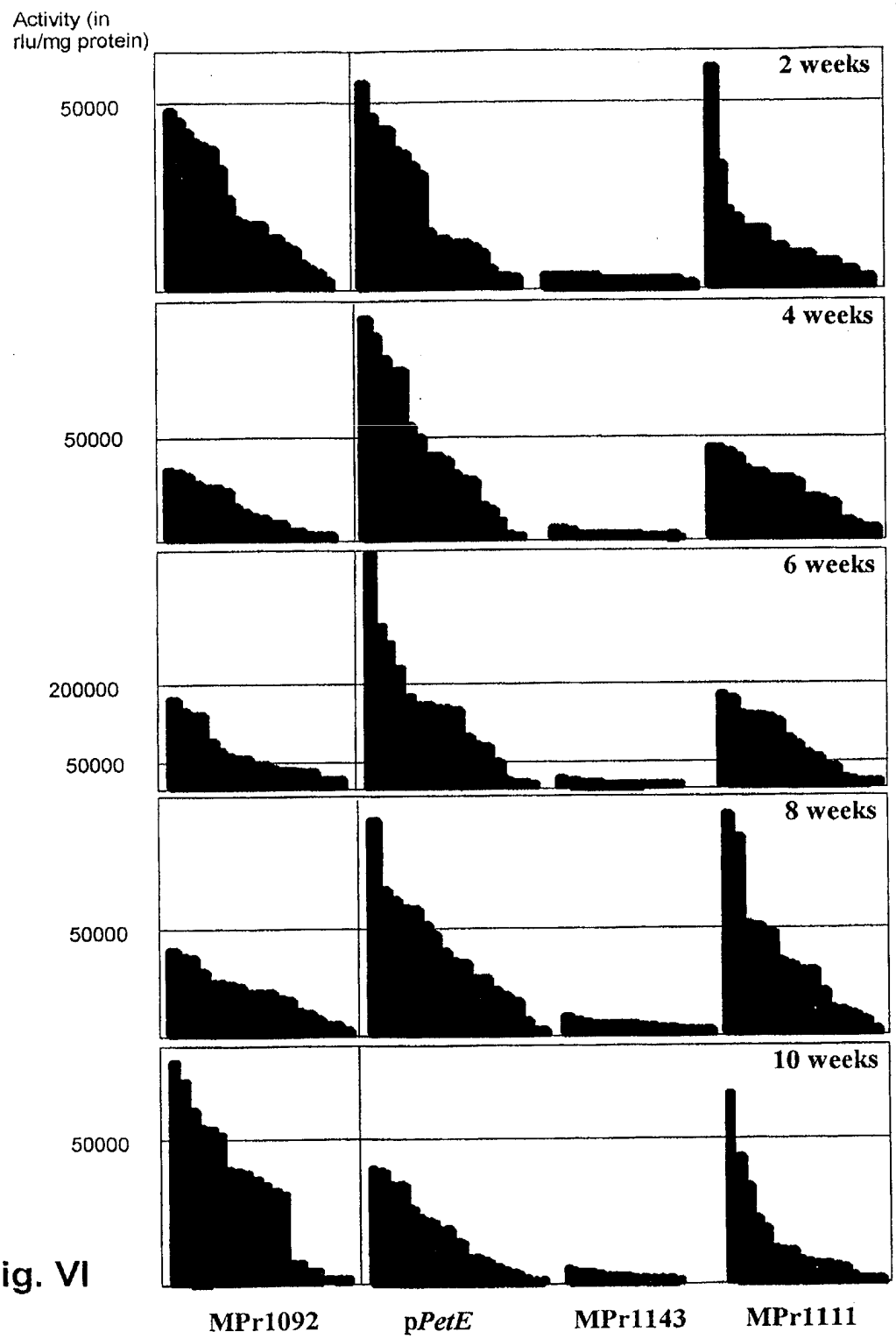
Fig. V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4 weeks
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p*PetE*
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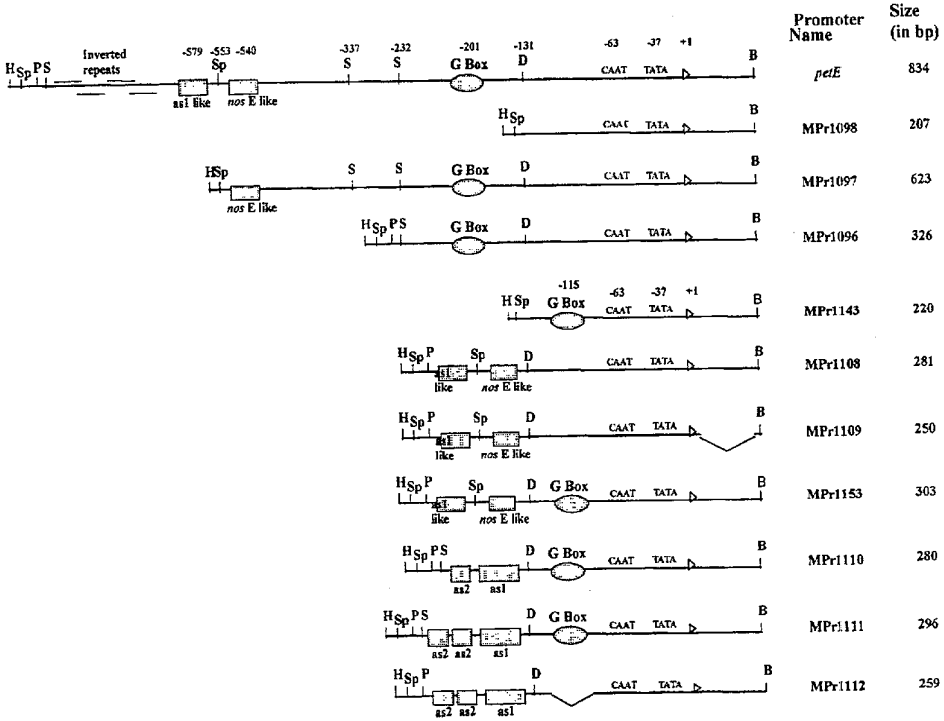
Fig. VI




| Promoter Name | Size (in bp) |
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| MPr1097 | 623 |
| MPr1096 | 326 |
| MPr1143 | 220 |
| MPr1108 | 281 |
| MPr1109 | 250 |
| MPr1153 | 303 |
| MPr1110 | 280 |
| MPr1111 | 296 |
| MPr1112 | 259 |